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(54) Title: REGULATION OF GENE EXPRESSION USING SINGLE-CHAIN, MONOMERIC, LIGAND DEPENDENT POLYPEPTIDE SWITCHES

(57) Abstract: Single chain, monomeric polypeptide gene switches are provided. The gene switches include ligand binding domains and at least one functional domain. Preferred functional domains are DNA binding domains and transcriptional regulating domains. Methods of regulating gene function using the switches are also provided.

Regulation of Gene Expression Using Single-Chain, Monomeric, Ligand Dependent Polypeptide Switches

5 Technical Field of the Invention

The field of this invention is regulation of transcription. More particularly, the present invention pertains to polypeptides that can activate or repress transcription in a small molecule ligand-dependent manner.

10 Background of the Invention

Designed transcription factors with defined target specificity and regulatory function provide invaluable tools for basic and applied research, and for gene therapy. Accordingly, the design of sequence-specific DNA binding domains has been the subject of intense interest for the last two decades. Of the many classes of DNA binding proteins studied, the modular Cys₂-His₂ zinc finger DNA binding motif has shown the most promise for the production of proteins with tailored DNA binding specificity. The novel architecture of this class of proteins provides for the rapid construction of gene-specific targeting devices. Polydactyl zinc finger proteins are most readily prepared by assembly of modular zinc finger domains recognizing predefined three-nucleotide sequences (See, e.g., Segal, D. J., Dreier, B., Beerli, R. R., and Barbas, C. F., III (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2758-2763; Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633; and Beerli, R. R., Dreier, B., and Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* **97**, 1495-1500). Polydactyl proteins can be assembled using variable numbers of zinc finger domains of varied specificity providing DNA binding proteins that not only recognize novel sequences but also sequences of varied length. By combining six zinc finger domains, proteins have been produced that recognize 18 contiguous base pairs of DNA sequence, a DNA address sufficiently complex to specify any locus in the 4 billion-base pair human genome (or any other genome). Fusion of polydactyl zinc finger proteins of this type to activation or repression domains provides

transcription factors that efficiently and specifically modulate the expression of both transgenes and endogenous genes (Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633; and Beerli, R. R., Dreier, B., and Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* **97**, 1495-1500).

While the availability of designed transcription factors with tailored DNA binding specificities provides novel opportunities in transcriptional regulation, additional applications would be available to ligand-dependent transcription factors. Designer zinc finger proteins dependent on small molecule inducers 5 would have a number of applications, both for the regulation of endogenous genes, and for the development of inducible expression systems for the regulation of transgenes. Natural transcription factors are regulated by a number of different mechanisms, including posttranslational modification such as phosphorylation 10 (Janknecht, R., and Hunter, T. (1997) *EMBO J* **16**, 1620-1627; Darnell, J. E., Jr. 15 (1997) *Science* **277**, 1630-1635), or by ligand binding. The prototype ligand-activated transcription factors are members of the nuclear hormone receptor family, including the receptors for sex steroids or adrenocorticoids (Carson-Jurica, M. A., Schrader, W. T., and O'Malley, W. (1990) *Endocrine Reviews* **11**, 201-220; Evans, R. M. (1988) *Science* **240**, 889-895). These receptors are held 20 inactive in the absence of hormone, by association with a number of inactivating factors including hsp90 (Pratt, W. B., and Toft, D. O. (1997) *Endocrine Rev.* **18**, 306-360). Upon ligand binding, nuclear hormone receptors dissociate from the inactivating complex, dimerize, and become able to bind DNA and activate 25 transcription (Carson-Jurica, M. A., Schrader, W. T., and O'Malley, W. (1990) *Endocrine Reviews* **11**, 201-220; Evans, R. M. (1988) *Science* **240**, 889-89512-14; and Pratt, W. B., and Toft, D. O. (1997) *Endocrine Rev.* **18**, 306-360). Significantly, not only hormone binding but also inactivation and dimerization functions reside 30 within the ligand binding domain (LBD) of these proteins (Beato, M. (1989) *Cell* **56**, 335-344). This fact has been exploited experimentally and steroid hormone receptor LBDs have found wide use as tools to render heterologous proteins hormone-dependent.

In particular, the estrogen receptor (ER) LBD has been used to render the functions of c-Myc (Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. (1989) *Nature* **340**, 66-68), c-Fos (Superti-Furga, G., Bergers, G., Picard, D., and Busslinger, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5114-5118), and even the 5 cytoplasmic kinase c-Raf (. Samuels, M. L., Weber, M. J., Bishop, J. M., and McMahon, M. (1993) *Mol. Cell. Biol.* **13**, 6241-6252) hormone-dependent. To develop an inducible expression system for use in basic research and gene therapy, the availability of ligand-dependent transcriptional regulators is a prerequisite. Preferentially, these regulators would be activated by a small 10 molecule inducer with no other biological activity, bind specific sequences present only in the target promoter, and have low immunogenicity. A number of ligand-regulated artificial transcription factors have been generated by various means, using functional domains derived from either prokaryotes (Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5547-5551 20. Gossen, M., 15 Freundlieb, S., Bender, G., Müller, G., Hillen, W., and Bujard, H. (1995) *Science* **268**, 1766-1769 21. Labow, M. A., Baim, S. B., Shenk, T., and Levine, A. J. (1990) *Mol. Cell. Biol.* **10**, 3343-3356 22. Baim, S. B., Labow, M. A., Levine, A. J., and Shenk, T. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5072-5076) or eukaryotes (Christopherson, K. S., Mark, M. R., Bajaj, V., and Godowski, P. J. (1992) *Proc. 20 Natl. Acad. Sci. USA* **89**, 6314-6318 24. No, D., Yao, T.-P., and Evans, R. M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 3346-3351 25. Wang, Y., O'Malley, B. W., Jr., Tsai, S., and O'Malley, B. W. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8180-8184 25 Beerli *et al.* -35-26. Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) *Gene Therapy* **4**, 432-441 27. Braselmann, S., Graninger, P., and Busslinger, M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1657-1661 28. Louvion, J. F., Havaux-Copf, B., and Picard, D. (1993) *Gene* **131**, 129-134 29. Rivera, V. M., Clackson, T., Natesan, S., Pollock, R., Amara, J. F., Keenan, T., Magari, S. R., Phillips, T., Courage, N. L., Cerasoli, F., Jr., Holt, D. A., and Gilman, M. (1996) *Nature. Med.* **2**, 1028-1032).

30 Of the functional domains derived from eukaryotic proteins, nuclear hormone receptor LBDs have been the most widely used. In particular, regulators

based on the Gal4 DNA binding domain (DBD) fused to a human ER
(Braselmann, S., Graninger, P., and Busslinger, M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1657-1661; Louvion, J. F., Havaux-Copf, B., and Picard, D. (1993) *Gene* **131**, 129-134) or progesterone receptor (PR) LBD; (Wang, Y., O'Malley, B. W.,
5 Jr., Tsai, S., and O'Malley, B. W. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8180-8184;
Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) *Gene Therapy* **4**, 432-441), as well as the ecdysone-inducible system based on the
Drosophila ecdysone receptor (EcR) and the mammalian retinoid X receptor (RXR)
(Christopherson, K. S., Mark, M. R., Bajaj, V., and Godowski, P. J. (1992) *Proc.
10 Natl. Acad. Sci. USA* **89**, 6314-6318; No, D., Yao, T.-P., and Evans, R. M. (1996)
Proc. Natl. Acad. Sci. USA **93**, 3346-3351) have been described. Compared to the
heterodimeric EcR/RXR system, regulators based on the ER and PR LBDs have
the important advantage that they function as homodimers and require the
delivery of only one cDNA. However, while ecdysone has no known biological
15 effect on mammalian cells, estrogen and progesterone will elicit a biological
response in cells or tissues that express the endogenous steroid receptors. With the
availability of a mutated ER and a truncated PR LBDs that have lost
responsiveness to their natural ligands but not to synthetic antagonists such as 4-
hydroxytamoxifen (4-OHT) (Littlewood, T. D., Hancock, D. C., Danielian, P. S.,
20 Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* **23**, 1686-1690) or RU486
(Vegeto, E., Allan, G. F., Schrader, W. T., Tsai, M.-J., McDonnell, D. P., and
O'Malley, B. W. (1992) *Cell* **69**, 703-713), respectively, this is no longer of great
concern. Thus, steroid hormone receptor LBD-based inducible expression systems
can be developed that function independently of the endogenous steroid receptors.
25 To date, this has been shown for the PR LBD through the development of an
RU486-inducible expression system based on the Gal4 DBD (Wang, Y.,
O'Malley, B. W., Jr., Tsai, S., and O'Malley, B. W. (1994) *Proc. Natl. Acad. Sci.
USA* **91**, 8180-8184; Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S.
Y. (1997) *Gene Therapy* **4**, 432-441). An inducible expression system based on a
30 point-mutated (G525R) ER LBD (Littlewood, T. D., Hancock, D. C., Danielian,
P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* **23**, 1686-1690) that

has lost the responsiveness to estrogen but not the antagonist 4-OHT has not been described to date. Designed zinc finger proteins have a number of advantages as compared to other DBDs, including the one derived from Gal4, since the ability to engineer DNA binding specificities allows ligand-dependent regulators to be
5 directed to any desired artificial or natural promoter. Here we explore the utility of fusion proteins between designed zinc finger proteins and nuclear hormone receptor LBDs for the inducible control of gene expression.

Brief Summary of the Invention

10 In one embodiment, the present invention provides a non-naturally occurring polypeptide that contains two ligand binding domains operatively linked to each other and a first functional domain operatively linked to one of the ligand binding domains. The ligand binding domains are preferably covalently linked to each other. More preferably, the two binding domains are covalently
15 linked by means of a peptide linker that contains from about 10 to about 40 amino acid residues, preferably from about 15 to about 35 amino acid residues and, more preferably from about 18 to about 30 amino acid residues.

In one embodiment, the ligand binding domains are derived from nuclear hormone receptors. The ligand binding domains can be derived from the same or
20 different nuclear hormone receptors. Exemplary and preferred nuclear hormone receptors are steroid hormone receptors such as an estrogen receptor, a progesterone receptor, an ecdisone receptor and a retinoid X receptor.

The first functional domain can be any domain that alters the function or activity of a target nucleotide. In one embodiment, the first functional domain is a
25 nucleotide binding domain. Preferably, the nucleotide binding domain is a DNA binding domain. The DNA binding domain preferably contains at least one zinc finger DNA binding motif, more preferably from two to twelve zinc finger DNA binding motifs and, even more preferably from three to six zinc finger DNA binding motifs. In one embodiment, the zinc finger DNA binding motifs
30 specifically bind to a nucleotide sequence of the formula (GNN)₁₋₆, where G is guanidine and N is any nucleotide. In another embodiment, the first functional

domain is a transcriptional regulating domain such as a transcription activation domain or a transcription repression domain.

In still another embodiment, the polypeptide gene switch contains a second functional domain. In accordance with this embodiment, a preferred first 5 functional domain is a nucleotide binding domain and the second functional domain is a transcriptional regulating domain.

In one embodiment, a polypeptide of this invention includes (a) a DNA binding domain having from three to six zinc finger DNA binding motifs; (b) a first ligand binding domain derived from a retinoid X receptor operative linked to 10 the DNA binding domain, a second ligand binding domain derived from an ecdyzone receptor linked to the first ligand binding domain with a peptide spacer of from 18 to 36 amino acid residues; and (c) a transcription regulating domain operatively linked to the second binding domain.

In still another embodiment, a polypeptide gene switch includes (a) a 15 DNA binding domain having from three to six zinc finger DNA binding motifs; (b) a first ligand binding domain derived from a progesterone receptor operatively linked to the DNA binding domain, a second ligand binding domain derived from a progesterone receptor linked to the first ligand binding domain with a peptide spacer of from 18 to 36 amino acid residues; and (c) a transcription regulating 20 domain operatively linked to the second ligand binding domain.

In another aspect, the present invention provides polynucleotides that encode a polypeptide gene switch of the invention, expression vectors containing such polynucleotides and cells containing such nucleotides.

Another aspect of this invention provides a process of regulating the 25 function of a target nucleotide that contains a defined sequence. The process includes the step of exposing the target nucleotide to a polypeptide of this invention in the presence of a ligand that binds at least one of the ligand binding domains of the polypeptide. In a related aspect, the present invention provides a process for regulating transcription (e.g., expression) of a target nucleotide (e.g., 30 gene). In accordance with that process a target nucleotide that contains a defined sequence is exposed to a polypeptide of this invention in the presence of a ligand

that binds to at least one of the ligand binding domains of that polypeptide. The polypeptide contains a nucleotide binding domain that specifically binds to the defined sequence in the target nucleotide. Where the polypeptide gene switch contains a transcription repression domain, regulating is repression. Where the 5 polypeptide gene switch contains a transcription activation domain, regulating is activation.

Brief Description of the Drawings

In the drawings that form a portion of the specification

10 **Figure 1** shows generation of designed zinc finger proteins with novel DNA binding specificity. *A*, amino acid sequence of the three-finger proteins B3 and N1. DNA recognition helix positions -2 to 6, shown in bold print, were grafted into the framework of the three finger protein Sp1C. The location of the antiparallel β sheets and the α helices, structural hallmarks of zinc finger 15 domains, are as indicated. DNA binding specificity of each finger is show on the left. F1-3, Finger 1-3. *B*, ELISA analysis of DNA binding specificity. Zinc finger proteins were expressed in *E. coli* as MBP fusions and purified. Specificity of binding was analyzed by measuring binding to immobilized biotinylated hairpin oligonucleotides containing the indicated 5'-(GNN)₃-3' sequences. Black bars, B3; 20 gray bars, N1. The maximal signals were normalized to 1. The K_D value for binding to the specific target sequence was measured by electrophoretic mobility shift assay and is labeled on top of the corresponding bars.

25 **Figure 2** shows regulation of gene expression by hormone-dependent, single-chain ER fusion constructs. *A*, structure of ER fusion proteins. E2C, six finger protein; L, flexible peptide linker. *B*, fusion proteins with a single ER-LBD bind as dimers. HeLa cells were cotransfected with a C7-ER-VP64 expression vector, and the indicated TATA luciferase reporter plasmids carrying either one or two C7 binding sites. 24 h after transfection, cells were either left untreated (-), or 100 nM 4-OHT was added (+). Luciferase activity in total cell extracts was 30 measured 48 h after transfection. Each bar represents the mean value (+/- SD) of duplicate measurements. C, control plasmid pcDNA3 that does not express a

fusion protein. *C, D*, regulation of transcription through a single binding site by fusion proteins with two ER-LBDs. HeLa cells were cotransfected with the indicated expression vectors and the E2C-TATA-luciferase reporter plasmid, carrying a single E2C binding site upstream of a TATA box. 4-OHT induction
5 and measurement of luciferase activity was carried out as described in *B*.

Figure 3 shows regulation of gene expression by hormone-dependent, single-chain RXR/EcR fusion constructs. *A*, structure of single-chain RXR/EcR fusion proteins. *B*, regulation of transcription through a single binding site. HeLa cells were cotransfected with the indicated expression vectors and the E2C-
10 TATA-luciferase reporter plasmid, carrying a single E2C binding site. 24 h after transfection, cells were either left untreated (-), or 5 µM Ponasterone A was added (+). Luciferase activity in total cell extracts was measured 48 h after transfection. Each bar represents the mean value (+/- SD) of duplicate measurements.
pcDNA3.1, control plasmid that does not express a fusion protein.

15 **Figure 4** shows the nucleotide (SEQ ID NO: 31) and amino acid residue sequence (SEQ ID NO: 32) of zinc finger binding domain B3B.

Figure 5 shows the nucleotide (SEQ ID NO: 33) and amino acid residue sequence (SEQ ID NO: 34) of zinc finger binding domain 2C7.

20 **Figure 6** shows the nucleotide (SEQ ID NO: 35) and amino acid residue sequence (SEQ ID NO: 36) of zinc finger binding domain B3C2.

Figure 7 shows the nucleotide (SEQ ID NO: 37) sequence of repression domain (KRAB-A)₂.

Figure 8 shows the nucleotide (SEQ ID NO: 38) sequence of repression domain (SID)₂.

25 **Figure 9** shows the nucleotide (SEQ ID NO: 39) and amino acid residue sequence (SEQ ID NO: 40) of polypeptide E2C-ER-L-ER-VP64.

Figure 10 shows the nucleotide (SEQ ID NO: 41) and amino acid residue sequence (SEQ ID NO: 42) of polypeptide E2C-ER-LL-ER-VP64.

Detailed Description of the Invention

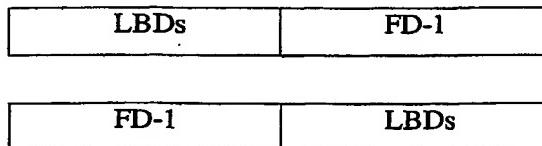
I. The Invention

The present invention provides polypeptide gene switches,
5 polynucleotides that encode such polypeptides, expression vectors that contain
such polynucleotides, cells that contain such expression vectors or
polynucleotides and processes for regulating target nucleotide function using such
polypeptides, polynucleotides and expression vectors. Unlike existing gene
switches that contain a single ligand binding domain together with a DNA binding
10 domain and/or a transcriptional regulating domain, polypeptide gene switches of
the present invention contain two ligand binding domains. Upon binding of the
ligand, an intramolecular configuration change occurs that allows for alignment of
the functional domains to the target gene of interest. An advantage of the present
gene switches, therefore, over existing gene switches is the need for only a single
15 molecular switch and a single expression vector for production of that switch.

II. Polypeptides

A polypeptide gene switch of the present invention includes at least three
components: two ligand binding domains (LBDs) and a first functional domain
(FD-1). The ligand binding domains are operatively linked to the first functional
20 domain such that the polypeptide, in the presence of a defined ligand that binds to
at least one of the ligand binding domains, can alter the function of nucleotide.
The domains can be arranged in any order. As shown below, the ligand binding
domains can be situated in either the amino- or carboxyl-terminal direction from
the first functional domain.

25



A polypeptide of this invention is non-naturally occurring. As used
herein, the term "non-naturally occurring" means, for example, one or more of the

following: (a) a peptide comprised of a non-naturally occurring amino acid sequence; (b) a peptide having a non-naturally occurring secondary structure not associated with the peptide as it occurs in nature; (c) a peptide which includes one or more amino acids not normally associated with the species of organism in
5 which that peptide occurs in nature; (d) a peptide which includes a stereoisomer of one or more of the amino acids comprising the peptide, which stereoisomer is not associated with the peptide as it occurs in nature; (e) a peptide which includes one or more chemical moieties other than one of the natural amino acids; or (f) an isolated portion of a naturally occurring amino acid sequence (e.g., a truncated
10 sequence). A polypeptide of this invention exists in an isolated form and purified to be substantially free of contaminating substances. A polypeptide is synthetic in nature. That is, the polypeptide is isolated and purified from natural sources or made *de novo* using techniques well known in the art.

15 A. Ligand Binding Domain (LBD)

Each LBD is an amino acid residue sequence that is capable of and binds a particular ligand. Binding of the ligand to the LBD alters the conformation/function of the polypeptide and allows for regulating a function of a target nucleotide. In the absence of ligand, the gene switch does not work to alter nucleotide function. At least one of the LBDs is capable of binding and binds a
20 particular ligand. Both LBDs can bind a particular ligand. Thus, the LBDs can be the same or different. Preferred LBDs are derived from nuclear hormone receptors such as steroid hormone receptors.

Exemplary and preferred steroid receptors that can serve as the source of ligand binding domains include the estrogen receptor (ER), progesterone receptor
25 (PR), glucocorticoid- α receptor, glucocorticoid- β receptor, mineralocorticoid receptor, androgen receptor, thyroid hormone receptor, retinoic acid receptor (RAR), retinoid X receptor (RXR), Vitamin D receptor, COUP-TF receptor, ecdysone receptor (EcR), Nurr-1 receptor and orphan receptors. A preferred EcR is derived either from *Drosophila melanogaster* (DE) or *Bombyx* (BE).

30 As is well known in the art, steroid hormone are composed of a DNA binding domain and a ligand binding domain. The DNA binding domain contains

the receptor regulating sequence and binds DNA and the ligand binding domain binds the specific biological compound (ligand) to activate the receptor. The term "ligand" refers to any compound which activates the receptor, usually by interaction with (binding) the ligand binding domain of the receptor. However,

5 ligands also include compounds that activate the receptor without binding. Where used in a polypeptide gene switch of the present invention, it is preferred that the ligand receptor domain be modified from its naturally occurring ligand, a ligand other than the naturally occurring ligand (e.g. steroid hormone). Means of altering or derivatizing naturally occurring nuclear hormone receptor ligand binding

10 domains to alter the binding specificity are well known in the art (See, e.g. United States Patent Nos. 5,874,534 and 5,599,904 the disclosures of which are incorporated herein by reference). Similarly, means for altering the estrogen receptor to change its bind affinity have reported [See, e.g. Littlewood et al., *Nucleic Acids Res.*, 3(10):1686-1690,1995].

15 The term "naturally occurring ligand" refers to compounds that are normally not found in animals or humans and which bind to the ligand binding domain of a receptor. The ligand can also be a "non-native ligand", a ligand that is not naturally found in the specific organism (man or animal) in which gene therapy is contemplated. For example, certain insect hormones such as ecdysone

20 are not found in humans. This is an example of a non-native hormone to the animal or human.

 Examples of non-natural ligands, anti-hormones and non-native ligands include the following: 11 β -(4-dimethylaminophenyl)-17 β -hydroxy-17 α -propinyl-4,9-e stradiene-3-one (Ru38486 or Mifepestone); 11 β -(4-dimethylaminophenyl)-

25 17 α -hydroxy-17 β -(3-hydroxypropyl)-13 α -methyl-4,9-gonadiene-3-one (ZK98299 or Onapristone); 11 β -(4-acetylphenyl)-17 β -hydroxy-17 α -(1-propinyl)-4,9-estradiene-3-one (ZK112993); 11 β -(4-dimethylaminophenyl)-17 β -hydroxy-17 α -(3-hydroxy-1 (Z)-propenyl-estra-4,9-diene-3-one (ZK98734); (7 β 11 β 17 β)-11-(4-dimethylaminophenyl)-7-methyl-4',5' dihydrospiroy'ester-4,9-diene-

30 17,2'(3'H)-furan!-3-one (Org31806); (11 β ,14 β ,17 α)-4',5'-dihydro-11-(4-dimethylaminophenyl)y'spi rostra-4,9-diene-17,2'(3'H)-furan!-3-one (Org31376);

5-alpha-pregnane-3,2-dione. Additional non-natural ligands include, in general, synthetic non-steroidal estrogenic or anti-estrogenic compounds, broadly defined as selective estrogen receptor modulators (SERMS). Exemplary compounds include, but are not limited to, tamoxifen and raloxifene,

5 Exemplary and preferred ligands for use with various ligand binding domains are (1) EcR: Ponasterone a, Muristerone A, GS-E (Invitrogen), Tebufenocide; (2) ER: estrogen antagonists such as 4-hydroxy-tamoxifen, ICI 164384, RU 54876, Raloxifene; and (3) PR: progesterone antagonists such as RU 486, RU 38486, and Onapristone.

10 An especially preferred LBD derived from a progesterone receptor comprises amino acid residues 645-914 from the human progesterone receptor. An exemplary LBD derived from an estrogen receptor comprises amino acid residues 282-599 from the mouse G225R mutant.

15 The two LBDs are separated by an amino acid residue sequence linker that contains from about 10 to about 50 amino acid residues. Preferably, the spacer contains from about 15 to about 40 amino acid residues and, more preferably, from about 18 to about 35 amino acid residues. Exemplary and preferred spacers contain 18 (L), 30 (LL), or 36 (LLL) amino acid residues.

B. Functional Domains

20 A second component of a present polypeptide is a functional domain. As used herein, the term "functional domain" and its grammatical equivalents, means an amino acid residue sequence that binds to, alters the structure of, and/or alters the function of, a nucleotide. Exemplary such functional domains include nucleotide binding domains, transcriptional regulating domains (e.g. transcription activation domains and transcription repression domains) and domains having nuclease activity. Such domains are well known in the art.

1. Nucleotide Binding Domains

25 A functional domain of a polypeptide can be a nucleotide binding domain: a sequence of amino acid residues that recognize and bind to a defined nucleotide sequence. The target nucleotide sequence can be an RNA sequence or, preferably, a DNA sequence. Amino acid residue sequences that recognize and

bind to defined DNA sequences are well known in the art (e.g., GAL4). Any such DNA binding peptide can be used as a DNA binding domain of a polypeptide gene switch of this invention. It is preferred, however, that the DNA binding domain of a present gene switch be one or more DNA binding zinc finger motifs.

5 Such zinc finger DNA binding motifs are well known in the art (See, e.g., PCT Patent Application Nos. WO95/19421 and WO 98/54311, the disclosures of which are incorporated herein by reference). A DNA binding domain of a polypeptide gene switch of this invention, thus, preferably includes a multiple finger, polydactyl, zinc finger peptide that is designed to bind specific nucleotide
10 target sequences.

The present disclosure is based on the recognition of the structural features unique to the Cys₂-His₂ zinc finger domain consist of a simple ββα fold of approximately 30 amino acids in length. Structural stability of this fold is achieved by hydrophobic interactions and by chelation of a single zinc ion by the
15 conserved Cys₂-His₂ residues (Lee, M.S., Gippert, G.P., Soman, K.V., Case, D.A. & Wright, P.E. (1989) *Science* **245**, 635-637). Nucleic acid recognition is achieved through specific amino acid side chain contacts originating from the α-helix of the domain, which typically binds three base pairs of DNA sequence
(Pavletich, N. P. & Pabo, C.O. (1991) *Science* **252**, 809-17, Elrod-Erickson, M.,
20 Rould, M.A., Nekludova, L. & Pabo, C.O. (1996) *Structure* **4**, 1171-1180). Unlike other nucleic acid recognition motifs, simple covalent linkage of multiple zinc finger domains allows the recognition of extended asymmetric sequences of DNA.

Studies of natural zinc finger proteins have shown that three zinc finger
25 domains can bind 9 bp of contiguous DNA sequence (Pavletich, N.P. & Pabo, C.O. (1991) *Science* **252**, 809-17., Swirnoff, A.H. & Milbrandt, J. (1995) *Mol. Cell. Biol.* **15**, 2275-87). Whereas recognition of 9 bp of sequence is insufficient to specify a unique site within even the small genome of *E.coli*, polydactyl proteins containing six zinc fingers domains can specify 18-bp recognition (Liu,
30 Q., Segal, D.J., Ghirardi, J.B. & Barbas III, C.F. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 5525-5530). With respect to the development of a universal system for gene

control, and 18-bp address can be sufficient to specify a single site within all known genomes. And their efficacy in gene activation and repression within living human cells has recently been shown (Liu, Q., Segal, D.J., Ghiara, J.B. & Barbas III, C.F. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 5525-5530).

5 The zinc finger-nucleotide binding peptide domain can be derived or produced from a wild type zinc finger protein by truncation or expansion, or as a variant of the wild type-derived polypeptide by a process of site directed mutagenesis, or by a combination of the procedures. The term "truncated" refers to a zinc finger-nucleotide binding polypeptide that contains less than the full 10 number of zinc fingers found in the native zinc finger binding protein or that has been deleted of non-desired sequences. For example, truncation of the zinc finger-nucleotide binding protein TFIIIA, which naturally contains nine zinc fingers, might be a polypeptide with only zinc fingers one through three. Expansion refers to a zinc finger polypeptide to which additional zinc finger modules have been 15 added. For example, TFIIIA may be extended to 12 fingers by adding 3 zinc finger modules from more than one wild type polypeptide, thus resulting in a "hybrid" zinc finger-nucleotide binding polypeptide.

The term "mutagenized" refers to a zinc finger derived-nucleotide binding polypeptide that has been obtained by performing any of the known methods for 20 accomplishing random or site-directed mutagenesis of the DNA encoding proteins. For instance, in TFIIIA, mutagenesis can be performed to replace non-conserved residues in one or more of the repeats of the consensus sequence. Truncated zinc finger-nucleotide binding proteins can also be mutagenized. Examples of known zinc finger-nucleotide binding proteins can also be 25 mutagenized. Examples of known zinc finger-nucleotide binding polypeptides that can be truncated, expanded, and/or mutagenized according to the present invention in order to inhibit the function of a nucleotide sequence containing a zinc finger-nucleotide binding motif includes TFIIIA and zif268. Other zinc finger-nucleotide binding proteins will be known to those of skill in the art.

30 A zinc finger DNA binding domain can be made using a variety of standard techniques well known in the art. Phage display libraries of zinc finger

proteins were created and selected under conditions that favored enrichment of sequence specific proteins. Zinc finger domains recognizing a number of sequences required refinement by site-directed mutagenesis that was guided by both phage selection data and structural information.

5 A DNA binding domain used in a polypeptide of this invention is preferably a zinc finger-nucleotide binding peptide that binds to a (GNN)₁₋₆ nucleotide sequence. Zinc fingers that bind specifically to (GNN)₁₋₆ have been described in United States Patent Application Serial Number 09/173,941, filed October 16, 1998 (the disclosure of which is incorporated herein by reference).

10 Exemplary and preferred zinc finger DNA binding domains are designated herein as E2C, C7, B3B, 2C7, B3C2 and N1. A detailed description of the preparation of polypeptide gene switches containing zinc finger DNA binding domains can be found hereinafter in the Examples. The amino acid residue and encoding nucleotide sequences for B3B, 2C7 and B3C2 are shown in FIGs. 4-6, 15 respectively.

2. Transcription Regulating Domains

A transcription regulating domain refers to a peptide, which acts to activate or repress transcription of a target nucleotide (e.g., gene). Transcriptional activation domains are well known in the art (See, e.g., Seipel et al., (1992) 20 *EMBO J.*, 11:4961-4968). Exemplary and preferred transcription activation domains include VP16, TA2, VP64, STAT6, relA, TAF-1, TAF-2, TAU-1 and TAU-2. Especially preferred activation domains for use in the present invention are VP16 and VP64. Means for linking VP16 and VP64 to ligand binding domains are set forth hereinafter in the Examples.

25 Transcriptional repressor domains are also well known in the art. Exemplary and preferred such transcriptional repressors are ERD, KRAB, SID, histone deacetylase, DNA, methylase, and derivatives, multimers and combinations thereof such as KRAB-ERD, SID-ERD, (KRAB)₂, (KRAB)₃, KRAB-A, (KRAB-A)₂, (SID)₂, (KRAB-A)-SID and SID-(KRAB-A). A first 30 repressor domain can be prepared using the Krappel-associated box (KRAB) domain (Margolin et al., 1994). This repressor domain is commonly found at the

N-terminus of zinc finger proteins and presumably exerts its repressive activity on TATA-dependent transcription in a distance- and orientation-independent manner, by interacting with the RING finger protein KAP-1. One can utilize the KRAB domain found between amino acids 1 and 97 of the zinc finger protein KOX1.

- 5 Finally, to explore the utility of histone deacetylation for repression, amino acids 1 to 36 of the Mad mSIN2 interaction domain (SID) can be fused to another domain (Ayer *et al.*, 1996). This small domain is found at the N-terminus of the transcription factor Mad and is responsible for mediating its transcriptional repression by interacting with mSIN3, which in turn interacts the co-repressor N-
10 CoR and with the histone deacetylase mRPD1.

The amino acid residue and nucleotide encoding sequences of preferred transcriptional repression domains (KRAB-A)₂ and (SID)₂ are shown in FIGs 7 and 8, respectively. Means for linking repression domains to ligand binding domains as well as exemplary polypeptide gene switches containing repression
15 domains are set forth hereinafter in the Examples.

3. Polypeptide Gene Switches

A polypeptide of this invention, in one embodiment, comprises two ligand binding domains and a first functional domain. In another embodiment, a polypeptide gene switch comprises two ligand binding domains, a first functional
20 domain and a second functional domain. These domains can exist in any order as shown below.

In a preferred embodiment the two ligand binding domains (LBDs) are located directly adjacent to one another, ie. they are "serially connected" within the monomeric polypeptide gene switch of the invention and are not separated by
25 a functional domain of the invention. The serially connected LBDs may be separated from one another by a linker molecule, such as for example a polypeptide linker molecule.

In a preferred embodiment the two LBDs are located between two functional domains (FDs) of the invention, wherein one functional domain is a
30 Transcription Regulating Domain (TRD) and the other functional domain is a Nucleotide Binding Domain (NBD).

In one particularly preferred embodiment the monomeric polypeptide gene switch of the invention consists of two FDs and two LBDs in the sequential order FD-1 / LBD-1 / LBD-2 / FD-2. Preferredly, in this embodiment, one functional domain is a TRD and the other functional domain is a NBD.

5 Preferredly, the NBD employed in the monomeric polypeptide gene switch of the invention includes 6 zinc finger binding motifs. As further described in the examples hereinbelow, a 6 zinc finger NBD employed in a monomeric polypeptide gene switch allows for the recognition of a unique 18bp nucleic acid sequence, which may be symmetric or asymmetric.

10

LBDs	FD-1	FD-2
FD-1	FD-2	LBDs
FD-1	LBDs	FD-2
FD-2	LBDs	FD-1
FD-2	FD-1	LBDs

A wide variety of polypeptide gene switches have been made. Exemplary such gene switches include (see above for definition of terms):

Gene Switches Using RXR, E2C, and Activation Domains

15 E2C-RXR-L-DE-VP64, E2C-RXR-LL-DE-VP64, E2C-RXR-LLL-DE-VP64, E2C-RXR-L-BE-VP64, E2C-RXR-LL-BE-VP64, E2C-RXR-LLL-BE-VP64, E2C-RXR-L-DE-VP16, E2C-RXR-LL-DE-VP16, E2C-RXR-LLL-DE-VP16, E2C-RXR-L-BE-VP16, E2C-RXR-LL-BE-VP16, E2C-RXR-LLL-BE-VP16;

20 Gene Switches Using RXR, 2C7, and Activation Domains

2C7-RXR-L-DE-VP64, 2C7-RXR-LL-DE-VP64, 2C7-RXR-LLL-DE-VP64, 2C7-RXR-L-BE-VP64, 2C7-RXR-LL-BE-VP64, 2C7-RXR-LLL-BE-

VP64, 2C7-RXR-L-DE-VP16, 2C7-RXR-LL-DE-VP16, 2C7-RXR-LLL-DE-
VP16, 2C7-RXR-L-BE-VP16, 2C7-RXR-LL-BE-VP16, E2C-RXR-LLL-BE-
VP16;

Gene Switches Using RXR, B3B, and Activation Domains

5 B3B-RXR-L-DE-VP64, B3B-RXR-LL-DE-VP64, B3B-RXR-LLL-DE-
VP64, B3B 7-RXR-L-BE-VP64, B3B 7-RXR-LL-BE-VP64, B3B-RXR-LLL-BE-
VP64, B3B-RXR-L-DE-VP16, B3B-RXR-LL-DE-VP16, B3B-RXR-LLL-DE-
VP16, B3B-RXR-L-BE-VP16, B3B-RXR-LL-BE-VP16, B3B-RXR-LLL-BE-
VP16;

10 Gene Switches Using RXR, B3C2, and Activation Domains

 B3C2-RXR-L-DE-VP64, B3C2-RXR-LL-DE-VP64, B3C2-RXR-LLL-
DE-VP64, B3C2-RXR-L-BE-VP64, B3C2-RXR-LL-BE-VP64, B3C2-RXR-
LLL-BE-VP64, B3C2-RXR-L-DE-VP16, B3C2-RXR-LL-DE-VP16, B3C2-
RXR-LLL-DE-VP16, B3C2-RXR-L-BE-VP16, B3C2 B-RXR-LL-BE-VP16,
15 B3C2-RXR-LLL-BE-VP16;

Gene Switches Using RXR, E2C, and Repression Domains

 E2C-RXR-L-DE-(KRAB-A)2, E2C-RXR-LL-DE-(KRAB-A)2, E2C-
RXR-LLL-DE-(KRAB-A)2, E2C-RXR-L-BE-(KRAB-A)2, E2C-RXR-LL-BE-
(KRAB-A)2, E2C-RXR-LLL-BE-(KRAB-A)2, E2C-RXR-L-DE-(KRAB-A)2,
20 E2C-RXR-LL-DE-(KRAB-A)2, E2C-RXR-LLL-DE-(KRAB-A)2, E2C-RXR-L-
BE-(KRAB-A)2, E2C-RXR-LL-BE-(KRAB-A)2, E2C-RXR-LLL-BE-(KRAB-
A)2, E2C-RXR-L-DE-(SID)2, E2C-RXR-LL-DE-(SID)2, E2C-RXR-LLL-DE-
(SID)2, E2C-RXR-L-BE-(SID)2, E2C-RXR-LL-BE-(SID)2, E2C-RXR-LLL-BE-
(SID)2, E2C-RXR-L-DE-(SID)2, E2C-RXR-LL-DE-(SID)2, E2C-RXR-LLL-DE-
25 (SID)2, E2C-RXR-L-BE-(SID)2, E2C-RXR-LL-BE-(SID)2, E2C-RXR-LLL-BE-
(SID)2;

Gene Switches Using RXR, 2C7, and Repression Domains

 2C7-RXR-L-DE-(KRAB-A)2, 2C7-RXR-LL-DE-(KRAB-A)2, 2C7-RXR-
LLL-DE-(KRAB-A)2, 2C7-RXR-L-BE-(KRAB-A)2, 2C7-RXR-LL-BE-(KRAB-
A)2, 2C7-RXR-LLL-BE-(KRAB-A)2, 2C7-RXR-L-DE-(KRAB-A)2, 2C7-RXR-
LL-DE-(KRAB-A)2, 2C7-RXR-LLL-DE-(KRAB-A)2, 2C7-RXR-L-BE-(KRAB-

A)2, 2C7-RXR-LL-BE-(KRAB-A)2, E2C-RXR-LLL-BE-(KRAB-A)2, 2C7-RXR-L-DE-(SID)2, 2C7-RXR-LL-DE-(SID)2, 2C7-RXR-LLL-DE-(SID)2, 2C7-RXR-L-BE-(SID)2, 2C7-RXR-LL-BE-(SID)2, 2C7-RXR-LLL-BE-(SID)2, 2C7-RXR-L-DE-(SID)2, 2C7-RXR-LL-DE-(SID)2, 2C7-RXR-LLL-DE-(SID)2, 2C7-RXR-L-BE-(SID)2, 2C7-RXR-LL-BE-(SID)2, E2C-RXR-LLL-BE-(SID)2,n;

Gene Switches Using RXR, B3B, and Repression Domains

B3B-RXR-L-DE-(KRAB-A)2, B3B-RXR-LL-DE-(KRAB-A)2, B3B-RXR-LLL-DE-(KRAB-A)2, B3B 7-RXR-L-BE-(KRAB-A)2, B3B 7-RXR-LL-BE-(KRAB-A)2, B3B-RXR-LLL-BE-(KRAB-A)2, B3B-RXR-L-DE-(KRAB-A)2, B3B-RXR-LL-DE-(KRAB-A)2, B3B-RXR-LLL-DE-(KRAB-A)2, B3B-RXR-L-BE-(KRAB-A)2, B3B-RXR-LL-BE-(KRAB-A)2, B3B-RXR-LLL-BE-(KRAB-A)2, B3B-RXR-L-DE-(SID)2, B3B-RXR-LL-DE-(SID)2, B3B-RXR-LLL-DE-(SID)2, B3B 7-RXR-L-BE-(SID)2, B3B 7-RXR-LL-BE-(SID)2, B3B-RXR-LLL-BE-(SID)2, B3B-RXR-L-DE-(SID)2, B3B-RXR-LL-DE-(SID)2, B3B-RXR-LLL-DE-(SID)2, B3B-RXR-L-BE-(SID)2, B3B-RXR-LL-BE-(SID)2;

Gene Switches Using RXR, B3C2, and Repression Domains

B3C2-RXR-L-DE-(KRAB-A)2, B3C2-RXR-LL-DE-(KRAB-A)2, B3C2-RXR-LLL-DE-(KRAB-A)2, B3C2-RXR-L-BE-(KRAB-A)2, B3C2-RXR-LL-BE-(KRAB-A)2, B3C2-RXR-L-DE-(KRAB-A)2, B3C2-RXR-LL-DE-(KRAB-A)2, B3C2-RXR-LLL-DE-(KRAB-A)2, B3C2-RXR-L-BE-(KRAB-A)2, B3C2 B-RXR-LL-BE-(KRAB-A)2, B3C2-RXR-LLL-BE-(KRAB-A)2, B3C2-RXR-L-DE-(SID)2, B3C2-RXR-LL-DE-(SID)2, B3C2-RXR-LLL-DE-(SID)2, B3C2-RXR-L-BE-(SID)2, B3C2-RXR-LL-BE-(SID)2, B3C2-RXR-LLL-BE-(SID)2, B3C2-RXR-L-DE-(SID)2, B3C2-RXR-LL-DE-(SID)2, B3C2-RXR-LLL-DE-(SID)2, B3C2-RXR-L-BE-(SID)2, B3C2-RXR-LL-BE-(SID)2;

Gene Switches Using PR, E2C, and Activation Domains

E2C-PR-L-PR-VP64, E2C-PR-LL-PR-VP64, E2C-PR-LLL-PR-VP64, E2C-PR-L-PR-VP64, E2C-PR-LL-PR-VP64, E2C-PR-LLL-PR-VP64, E2C-PR-L-PR-VP16, E2C-PR-LL-PR-VP16, E2C-PR-LLL-PR-VP16, E2C-PR-L-PR-

VP16, E2C-PR-LL-PR-VP16, E2C-PR-LLL-PR-VP16;

Gene Switches Using PR, 2C7, and Activation Domains

2C7-PR-L-PR-VP64, 2C7-PR-LL-PR-VP64, 2C7-PR-LLL-PR-VP64,
2C7-PR-L-PR-VP64, 2C7-PR-LL-PR-VP64, 2C7-PR-LLL-PR-VP64, 2C7-PR-L-
5 PR-VP16, 2C7-PR-LL-PR-VP16, 2C7-PR-LLL-PR-VP16, 2C7-PR-L-PR-VP16,
2C7-PR-LL-PR-VP16, E2C-PR-LLL-PR-VP16;

Gene Switches Using PR, B3B, and Activation Domains

B3B-PR-L-PR-VP64, B3B-PR-LL-PR-VP64, B3B-PR-LLL-PR-VP64,
B3B 7-PR-L-PR-VP64, B3B 7-PR-LL-PR-VP64, B3B-PR-LLL-PR-VP64, B3B-
10 PR-L-PR-VP16, B3B-PR-LL-PR-VP16, B3B-PR-LLL-PR-VP16, B3B-PR-L-PR-
VP16, B3B-PR-LL-PR-VP16, B3B-PR-LLL-PR-VP16;

Gene Switches Using PR, B3C2, and Activation Domains

B3C2-PR-L-PR-VP64, B3C2-PR-LL-PR-VP64, B3C2-PR-LLL-PR-
VP64, B3C2-PR-L-PR-VP64, B3C2-PR-LL-PR-VP64, B3C2-PR-LLL-PR-VP64,
15 B3C2-PR-L-PR-VP16, B3C2-PR-LL-PR-VP16, B3C2-PR-LLL-PR-VP16,
B3C2-PR-L-PR-VP16, B3C2 B-PR-LL-PR-VP16, B3C2-PR-LLL-PR-VP16;

Gene Switches Using PR, E2C, and Repression Domains

E2C-PR-L-PR-(KRAB-A)2, E2C-PR-LL-PR-(KRAB-A)2, E2C-PR-LLL-
PR-(KRAB-A)2, E2C-PR-L-PR-(KRAB-A)2, E2C-PR-LL-PR-(KRAB-A)2,
20 E2C-PR-LLL-PR-(KRAB-A)2, E2C-PR-L-PR-(KRAB-A)2, E2C-PR-LL-PR-
(KRAB-A)2, E2C-PR-LLL-PR-(KRAB-A)2, E2C-PR-L-PR-(KRAB-A)2, E2C-
PR-LL-PR-(KRAB-A)2, E2C-PR-LLL-PR-(KRAB-A)2, E2C-PR-L-PR-(SID)2,
E2C-PR-LL-PR-(SID)2, E2C-PR-LLL-PR-(SID)2, E2C-PR-L-PR-(SID)2, E2C-
PR-LL-PR-(SID)2, E2C-PR-LLL-PR-(SID)2, E2C-PR-L-PR-(SID)2, E2C-PR-
25 LL-PR-(SID)2, E2C-PR-LLL-PR-(SID)2, E2C-PR-L-PR-(SID)2, E2C-PR-LL-
PR-(SID)2, E2C-PR-LLL-PR-(SID)2;

Gene Switches Using PR, 2C7, and Repression Domains

2C7-PR-L-PR-(KRAB-A)2, 2C7-PR-LL-PR-(KRAB-A)2, 2C7-PR-LLL-
PR-(KRAB-A)2, 2C7-PR-L-PR-(KRAB-A)2, 2C7-PR-LL-PR-(KRAB-A)2, 2C7-
30 PR-LLL-PR-(KRAB-A)2, 2C7-PR-L-PR-(KRAB-A)2, 2C7-PR-LL-PR-(KRAB-
A)2, 2C7-PR-LLL-PR-(KRAB-A)2, 2C7-PR-L-PR-(KRAB-A)2, 2C7-PR-LL-

PR-(KRAB-A)2, E2C-PR-LLL-PR-(KRAB-A)2, 2C7-PR-L-PR-(SID)2, 2C7-
PR-LL-PR-(SID)2, 2C7-PR-LLL-PR-(SID)2, 2C7-PR-L-PR-(SID)2, 2C7-PR-LL-
PR-(SID)2, 2C7-PR-LLL-PR-(SID)2, 2C7-PR-L-PR-(SID)2, 2C7-PR-LL-PR-
(SID)2, 2C7-PR-LLL-PR-(SID)2, 2C7-PR-L-PR-(SID)2, 2C7-PR-LL-PR-(SID)2,
5 E2C-PR-LLL-PR-(SID)2,n;

Gene Switches Using PR, B3B, and Repression Domains

B3B-PR-L-PR-(KRAB-A)2, B3B-PR-LL-PR-(KRAB-A)2, B3B-PR-LLL-
PR-(KRAB-A)2, B3B 7-PR-L-PR-(KRAB-A)2, B3B 7-PR-LL-PR-(KRAB-A)2,
B3B-PR-LLL-PR-(KRAB-A)2, B3B-PR-L-PR-(KRAB-A)2, B3B-PR-LL-PR-
10 (KRAB-A)2, B3B-PR-LLL-PR-(KRAB-A)2, B3B-PR-L-PR-(KRAB-A)2, B3B-
PR-LL-PR-(KRAB-A)2, B3B-PR-LLL-PR-(KRAB-A)2, B3B-PR-L-PR-(SID)2,
B3B-PR-LL-PR-(SID)2, B3B-PR-LLL-PR-(SID)2, B3B 7-PR-L-PR-(SID)2, B3B
7-PR-LL-PR-(SID)2, B3B-PR-LLL-PR-(SID)2, B3B-PR-L-PR-(SID)2, B3B-PR-
LL-PR-(SID)2, B3B-PR-LLL-PR-(SID)2, B3B-PR-L-PR-(SID)2, B3B-PR-LL-
15 PR-(SID)2, B3B-PR-LLL-PR-(SID)2;

Gene Switches Using PR, B3C2, and Repression Domains

B3C2-PR-L-PR-(KRAB-A)2, B3C2-PR-LL-PR-(KRAB-A)2, B3C2-PR-
LLL-PR-(KRAB-A)2, B3C2-PR-L-PR-(KRAB-A)2, B3C2-PR-LL-PR-(KRAB-
A)2, B3C2-PR-LLL-PR-(KRAB-A)2, B3C2-PR-L-PR-(KRAB-A)2, B3C2-PR-
20 LL-PR-(KRAB-A)2, B3C2-PR-LLL-PR-(KRAB-A)2, B3C2-PR-L-PR-(KRAB-
A)2, B3C2 B-PR-LL-PR-(KRAB-A)2, B3C2-PR-LLL-PR-(KRAB-A)2, B3C2-
PR-L-PR-(SID)2, B3C2-PR-LL-PR-(SID)2, B3C2-PR-LLL-PR-(SID)2, B3C2-
PR-L-PR-(SID)2, B3C2-PR-LL-PR-(SID)2, B3C2-PR-LLL-PR-(SID)2, B3C2-
PR-L-PR-(SID)2, B3C2-PR-LL-PR-(SID)2, B3C2-PR-LLL-PR-(SID)2, B3C2-
25 PR-L-PR-(SID)2, B3C2 B-PR-LL-PR-(SID)2, B3C2-PR-LLL-PR-(SID)2;

Gene Switches Using ER, E2C, and Activation Domains

E2C-ER-L-ER-VP64, E2C-ER-LL-ER-VP64, E2C-ER-LLL-ER-VP64,
E2C-ER-L-ER-VP64, E2C-ER-LL-ER-VP64, E2C-ER-LLL-ER-VP64, E2C-ER-
L-ER-VP16, E2C-ER-LL-ER-VP16, E2C-ER-LLL-ER-VP16, E2C-ER-L-ER-
30 VP16, E2C-ER-LL-ER-VP16, E2C-ER-LLL-ER-VP16;

Gene Switches Using ER, 2C7, and Activation Domains

2C7-ER-L-ER-VP64, 2C7-ER-LL-ER-VP64, 2C7-ER-LLL-ER-VP64,
2C7-ER-L-ER-VP64, 2C7-ER-LL-ER-VP64, 2C7-ER-LLL-ER-VP64, 2C7-ER-
L-ER-VP16, 2C7-ER-LL-ER-VP16, 2C7-ER-LLL-ER-VP16, 2C7-ER-L-ER-
VP16, 2C7-ER-LL-ER-VP16, E2C-ER-LLL-ER-VP16;

5 Gene Switches Using ER, B3B, and Activation Domains

B3B-ER-L-ER-VP64, B3B-ER-LL-ER-VP64, B3B-ER-LLL-ER-VP64,
B3B 7-ER-L-ER-VP64, B3B 7-ER-LL-ER-VP64, B3B-ER-LLL-ER-VP64, B3B-
ER-L-ER-VP16, B3B-ER-LL-ER-VP16, B3B-ER-LLL-ER-VP16, B3B-ER-L-
ER-VP16, B3B-ER-LL-ER-VP16, B3B-ER-LLL-ER-VP16;

10 Gene Switches Using ER, B3C2, and Activation Domains

B3C2-ER-L-ER-VP64, B3C2-ER-LL-ER-VP64, B3C2-ER-LLL-ER-
VP64, B3C2-ER-L-ER-VP64, B3C2-ER-LL-ER-VP64, B3C2-ER-LLL-ER-
VP64, B3C2-ER-L-ER-VP16, B3C2-ER-LL-ER-VP16, B3C2-ER-LLL-ER-
VP16, B3C2-ER-L-ER-VP16, B3C2 B-ER-LL-ER-VP16, B3C2-ER-LLL-ER-

15 VP16;

Gene Switches Using ER, E2C, and Repression Domains

E2C-ER-L-ER-(KRAB-A)2, E2C-ER-LL-ER-(KRAB-A)2, E2C-ER-
LLL-ER-(KRAB-A)2, E2C-ER-L-ER-(KRAB-A)2, E2C-ER-LL-ER-(KRAB-
A)2, E2C-ER-LLL-ER-(KRAB-A)2, E2C-ER-L-ER-(KRAB-A)2, E2C-ER-LL-
ER-(KRAB-A)2, E2C-ER-LLL-ER-(KRAB-A)2, E2C-ER-L-ER-(KRAB-A)2,
E2C-ER-LL-ER-(KRAB-A)2, E2C-ER-LLL-ER-(KRAB-A)2, E2C-ER-L-ER-
(SID)2, E2C-ER-LL-ER-(SID)2, E2C-ER-LLL-ER-(SID)2, E2C-ER-L-ER-
(SID)2, E2C-ER-LL-ER-(SID)2, E2C-ER-LLL-ER-(SID)2, E2C-ER-L-ER-
(SID)2, E2C-ER-LL-ER-(SID)2, E2C-ER-LLL-ER-(SID)2, E2C-ER-L-ER-
(SID)2, E2C-ER-LL-ER-(SID)2, E2C-ER-LLL-ER-(SID)2;

Gene Switches Using ER, 2C7, and Repression Domains

2C7-ER-L-ER-(KRAB-A)2, 2C7-ER-LL-ER-(KRAB-A)2, 2C7-ER-LLL-
ER-(KRAB-A)2, 2C7-ER-L-ER-(KRAB-A)2, 2C7-ER-LL-ER-(KRAB-A)2,
2C7-ER-LLL-ER-(KRAB-A)2, 2C7-ER-L-ER-(KRAB-A)2, 2C7-ER-LL-ER-
30 (KRAB-A)2, 2C7-ER-LLL-ER-(KRAB-A)2, 2C7-ER-L-ER-(KRAB-A)2, 2C7-
ER-LL-ER-(KRAB-A)2, E2C-ER-LLL-ER-(KRAB-A)2, 2C7-ER-L-ER-(SID)2,

2C7-ER-LL-ER-(SID)2, 2C7-ER-LLL-ER-(SID)2, 2C7-ER-L-ER-(SID)2, 2C7-ER-LL-ER-(SID)2, 2C7-ER-LLL-ER-(SID)2, 2C7-ER-L-ER-(SID)2, 2C7-ER-LL-ER-(SID)2, 2C7-ER-LLL-ER-(SID)2, 2C7-ER-L-ER-(SID)2, 2C7-ER-LL-ER-(SID)2, E2C-ER-LLL-ER-(SID)2,n;

5 Gene Switches Using ER, B3B, and Repression Domains

B3B-ER-L-ER-(KRAB-A)2, B3B-ER-LL-ER-(KRAB-A)2, B3B-ER-LLL-ER-(KRAB-A)2, B3B 7-ER-L-ER-(KRAB-A)2, B3B 7-ER-LL-ER-(KRAB-A)2, B3B-ER-LLL-ER-(KRAB-A)2, B3B-ER-L-ER-(KRAB-A)2, B3B-ER-LL-ER-(KRAB-A)2, B3B-ER-LLL-ER-(KRAB-A)2, B3B-ER-L-ER-(KRAB-A)2,
10 B3B-ER-LL-ER-(KRAB-A)2, B3B-ER-LLL-ER-(KRAB-A)2, B3B-ER-L-ER-(SID)2, B3B-ER-LL-ER-(SID)2, B3B 7-ER-L-ER-(SID)2, B3B 7-ER-LL-ER-(SID)2, B3B-ER-LLL-ER-(SID)2, B3B-ER-L-ER-(SID)2, B3B-ER-LL-ER-(SID)2, B3B-ER-LLL-ER-(SID)2, B3B-ER-L-ER-(SID)2;
15 Gene Switches Using ER, B3C2, and Repression Domains

B3C2-ER-L-ER-(KRAB-A)2, B3C2-ER-LL-ER-(KRAB-A)2, B3C2-ER-LLL-ER-(KRAB-A)2, B3C2-ER-L-ER-(KRAB-A)2, B3C2-ER-LL-ER-(KRAB-A)2, B3C2-ER-LLL-ER-(KRAB-A)2, B3C2-ER-L-ER-(KRAB-A)2, B3C2-ER-LL-ER-(KRAB-A)2, B3C2-ER-LLL-ER-(KRAB-A)2, B3C2-ER-L-ER-(KRAB-A)2, B3C2 B-ER-LL-ER-(KRAB-A)2, B3C2-ER-LLL-ER-(KRAB-A)2, B3C2-ER-L-ER-(SID)2, B3C2-ER-LL-ER-(SID)2, B3C2-ER-LLL-ER-(SID)2, B3C2-ER-L-ER-(SID)2, B3C2-ER-LL-ER-(SID)2, B3C2-ER-LLL-ER-(SID)2, B3C2-ER-L-ER-(SID)2, B3C2 B-ER-LL-ER-(SID)2, B3C2-ER-LLL-ER-(SID)2.

25 The nucleotide (SEQ ID NO: 39) and amino acid residue sequence (SEQ ID NO: 40) of polypeptide E2C-ER-L-ER-VP64 are shown in FIG. 9. The nucleotide (SEQ ID NO: 41) and amino acid residue sequence (SEQ ID NO: 42) of polypeptide E2C-ER-LL-ER-VP64 are shown in Fig. 10.

30 III. Polynucleotides, Expression Vectors and Host Cells

In a related aspect, the present invention provides polynucleotides that

encode a polypeptide gene switch of this invention, expression vectors containing those polynucleotides, cells containing those polynucleotides and transformed cells containing those expression vectors. Vectors of primary utility for gene therapy include, but are not limited to human adenovirus vectors, adeno-
5 associated vectors, murine or lenti virus derived retroviral vectors, or a variety of non-viral compositions including liposomes, polymers, and other DNA containing conjugates. Such vector systems can be used to deliver the gene switches either *in vitro* or *in vivo*, depending on the vector system. With adenovirus, for instance, vectors can be administered intravenously to transduce the liver and other organs,
10 introduced directly into the lung, or into vascular compartments temporarily localized by ligation or other methods. Methods for constructing such vectors, and methods and uses for the described invention are known to those skilled in the field of gene therapy.

15 IV. Methods of Regulating Nucleotide Function

The present invention further provides a process for regulating the expression of a desired nucleotide sequence such as a gene. In accordance with the process, the target nucleotide sequence is exposed to an effective amount of a gene switch and a ligand, wherein the nucleotide binding domain of the gene
20 switch binds to a portion of the target nucleotide and wherein the ligand binds to at least one of the ligand binding domains of the gene switch. Exposure can occur *in vitro*, *in situ* or *in vivo*. The term "effective amount" means that amount that regulates transcription of a nucleotide (e.g. structural gene or translation of RNA).

The term "regulating" refers to the suppression, enhancement, or induction
25 of a function. For example, a polypeptide of the invention may modulate a promoter sequence by binding to a motif within the promoter, thereby enhancing or suppressing transcription of a gene operatively linked to the promoter nucleotide sequence. Alternatively, modulation may include inhibition of a gene where the polypeptide binds to the structural gene and blocks DNA dependent
30 RNA polymerase from reading through the gene, thus inhibiting transcription of the gene. Alternatively, modulation may include inhibition of translation of a

transcript.

The promoter region of a gene includes the regulatory elements that typically lie 5' to a structural gene. If a gene is to be activated, proteins known as transcription factors attach to the promoter region of the gene. This assembly 5 resembles an "on switch" by enabling an enzyme to transcribe a second genetic segment from DNA to RNA. In most cases the resulting RNA molecule serves as a template for synthesis of a specific protein; sometimes RNA itself is the final product.

Regulation of gene expression or transcription can be accomplished both 10 by exposing the target gene to a polypeptide switch of this invention or, preferably by transforming a cell that contains the target gene with an expression vector that contains a polynucleotide sequence that encodes a gene switch.

The Examples that follow illustrate particular embodiments of the present invention and are not limiting of the specification or claims in any way.

15

EXAMPLE 1: General Methods

Construction of zinc finger proteins. For the construction of the B3 and N1 zinc finger proteins, DNA recognition helices from the Zif268 Finger 2 variants pmGAA, pmGAC, pmGGA, pmGGG, and pGTA were utilized [Segal, 20 D. J., Dreier, B., Beerli, R. R., and Barbas, C. F., III (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2758-2763]. Three finger proteins binding the respective 9-bp target-sites were constructed by grafting the appropriate DNA recognition helices into the framework of the three finger protein Sp1C [Desjarlais, J. R., and Berg, J. M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2256-2260]; DNA fragments encoding the 25 two 3 finger proteins were assembled from 6 overlapping oligonucleotides as described [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633]. The three finger protein coding regions were then cloned into the bacterial expression vector pMal-CSS via Sfi1 digestion.

30 **Protein purification.** Maltose binding protein (MBP) fusion proteins were purified to >90% homogeneity using the Protein Fusion and Purification

System (New England Biolabs), except that Zinc Buffer A (ZBA; 10 mM Tris, pH7.5/90 mM KCl, 1 mM MgCl₂, 90 μM ZnCl₂)/1% BSA /5 mM DTT) was used as the column buffer. Protein purity and concentration were determined from Coomassie blue-stained 15% SDS-PAGE gels by comparison to BSA standards.

- 5 **ELISA analysis.** In 96-well ELISA plates, 0.2 μg of streptavidin (Pierce) was applied to each well for 1 hour at 37°C, then washed twice with water. Biotinylated target oligonucleotide (0.025 μg) was applied in the same manner. ZBA/3% BSA was applied for blocking, but the wells were not washed after incubation. All subsequent incubations were at room temperature. Starting with
10 2 μg purified MBP fusion protein in the top wells, 2-fold serial dilutions were applied in 1x binding buffer (ZBA/1% BSA/5 mM DTT/0.12 μg/μl sheared herring sperm DNA). The samples were incubated 1 hour at room temperature, followed by 10 washes with water. Mouse anti-maltose binding protein mAb (Sigma) in ZBA/1% BSA was applied to the wells for 30 minutes, followed by 10
15 washes with water. Goat anti -mouse IgG mAb conjugated to alkaline phosphatase (Sigma) was applied to the wells for 30 minutes, followed by 10 washes with water. Alkaline phosphatase substrate (Sigma) was applied, and the OD₄₀₅ was quantitated with SOFTmax 2.35 (Molecular Devices).

- 20 **Gel mobility shift assays.** Target oligonucleotides were labeled at their 3' ends with [³²P] and gel purified. Eleven 3-fold serial dilutions of protein were incubated in 20 μl binding reactions (1x Binding Buffer/10% glycerol/≈1 pM target oligonucleotide) for three hours at room temperature, then resolved on a 5% polyacrylamide gel in 0.5x TBE buffer. Quantitation of dried gels was performed using a PhosphorImager and ImageQuant software (Molecular Dynamics), and
25 the K_D was determined by Scatchard analysis.

- 30 **Reporter constructs for determining the optimal spacing and orientation of the two half-sites.** C7 dimer-TATA fragments were generated by PCR amplification with C7 dimer-TATA primers (5'-GAG GGT ACC GCG TGG GCG A₀₋₅ GCG TGG GCG AGT CGA CTC TAG AGG GTA TAT AAT GG-3'
(SEQ ID NO: 1) for direct repeats; 5'-GAG GGT ACC GCG TGG GCG A₀₋₅

CGC CCA CGC AGT CGA CTC TAG AGG GTA TAT AAT GG-3' (SEQ ID NO: 2) for inverted repeats; 5'-GAG GGT ACC CGC CCA CGC A₀₋₅ GCG TGG GCG AGT CGA CTC TAG AGG GTA TAT AAT GG-3' (SEQ ID NO: 3) for everted repeats) and GLprimer2 (5'-CTT TAT GTT TTT GGC GTC TTC C-3' (SEQ ID NO: 4); Promega), using p17x4TATA-luc (gift from S. Y. Tsai) as a template. PCR products were cloned into pGL3-Basic (Promega) via digestion with the restriction endonucleases Kpn1 and Nco1.

RU486- and Tamoxifen-inducible promoter constructs. 10xC7-TATA, 10xB3-TATA, and 10xN1-TATA fragments were assembled from two pairs of complementary oligonucleotides each and cloned into Sac1-Xma1 linearized pGL3-Basic (Promega), upstream of the firefly luciferase coding region, creating the plasmids 10xC7-TATA-luc, 10xB3-TATA-luc, and 10xN1-TATA-luc. To generate the 10xN1-TATA-lacZ reporter construct, the lacZ coding region was excised from p β gal-Basic (Clontech) and used to replace the luciferase coding region of 10xN1-TATA-luc via Hind3-BamH1 digestion.

Luciferase and β -gal reporter assays. For all transfections, HeLa cells were plated in 24-well dishes and used at a confluence of 40-60%. For luciferase reporter assays, 175 ng reporter plasmid (promotor constructs in pGL3 or, as negative control, pGL3-Basic) and 25 ng effector plasmid (zinc finger-steroid receptor fusions in pcDNA3 or, as negative control, empty pcDNA3) were transfected using the Lipofectamine reagent (Gibco BRL). After approximately 24 h, expression was induced by the addition of 10nM RU486 (Biomol), 100 nM 4-OHT (Sigma), or 5 mM Ponasterone A (Invitrogen). Cell extracts were prepared approximately 48 hours after transfection and assayed for luciferase activity using the Promega luciferase assay reagent in a MicroLumat LB96P luminometer (EG&G Berthold, Gaithersburg, MD). For dual reporter assays, 85ng luciferase reporter plasmid, 85ng b-gal reporter plasmid, and 15ng of each of the two effector plasmids were transfected. b-gal activity was measured using the luminescent b-galactosidase detection kit II (Clontech).

Zinc finger-steroid receptor fusion constructs with N-terminal effector domains. The VP16 coding region was PCR amplified from pcDNA3/C7-VP16 using the primers VPNhe-F (5'-GAG GAG GAG GAG GCT AGC GCC ACC ATG GGG CGC GCC GGC GCT CCC CCG ACC GAT GTC 5 AGC CTG-3') (SEQ ID NO: 5), and VPHind-B (5'-GAG GAG GAG GAG AAG CTT GTT AAT TAA ACC GTA CTC GTC AAT TCC AAG GGC ATC G-3') (SEQ ID NO: 6) or VPNLSHind-B (5'-GAG GAG GAG GAG AAG CTT AAC TTT GCG TTT CTT TTT CGG GTT AAT TAA ACC GTA CTC GTC AAT TCC AAG GGC ATC G-3') (SEQ ID NO: 7). The C7 coding region was 10 amplified from the same plasmid, using the primers C7Hind-F (5'-GAG GAG GAG AAG CTT GGG GCC ACG GCG GCC CTC GAG CCC TAT GC-3') (SEQ ID NO: 8), and C7Bam-B (5'-GAG GAG GGA TCC CCC TGG CCG GCC TGG CCA CTA GTT CTA GAG TC-3') (SEQ ID NO: 9) or C7NLSBam-B (5'-GAG GAG GGA TCC CCA ACT TTG CGT TTC TTT TTC GGC TGG 15 CCG GCC TGG CCA CTA GIT CTA GAG TC-3') (SEQ ID NO: 10). The human PR truncated LBD (aa645-914) was amplified from PAPCMVGL914VPc'-SV [Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) *Gene Therapy* 4, 432-441] using the primers PRBam-F (5'-GAG GAG GAG GGA TCC AGT CAG AGT TGT GAG AGC ACT GGA TGC 20 TG-3') (SEQ ID NO: 11) and PREco-B (5'-GAG GAG GAA TTC TCA AGC AAT AAC TTC AGA CAT CAT TTC TGG AAA TTC-3') (SEQ ID NO: 12). The VP16-C7-PR, VP16-NLS-C7-PR, and VP16-C7-NLS-PR coding regions were then assembled in pcDNA3.1(+)Zeo (Invitrogen) using the Nhe1, Hind3, BamH1, and EcoR1 restriction sites incorporated in the PCR primers. In the 25 resulting constructs, the C7 coding regions were flanked by two Sfi1 sites, and the VP16 coding regions by Asc1 and Pac1 sites. These restriction sites were introduced to facilitate the exchange of DBDs and effector domains, respectively.

To generate the VP16-C7-ER, VP16-NLS-C7-ER, and VP16-C7-NLS-ER constructs, the point-mutated murine ER LBD coding region (aa281-599, G525R) 30 was excised from pBabe/Myc-ER [Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* 23, 1686-1690], and

used to replace the PR LBD coding region via BamH1-EcoR1 restriction digestion.

To generate fusion constructs with B3 or N1 DBDs, C7 was replaced by the B3 or N1 coding regions via Sfi1 digestion. Fusion constructs containing a 5 VP64 effector domain were produced by replacing VP16 by the VP64 coding region via Asc1-Pac1 digestion.

Zinc finger-steroid receptor fusion constructs with C-terminal effector domains. The truncated human PR LBD was amplified from PAPCMVGL914VPc'-SV [Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and

10 Tsai, S. Y. (1997) *Gene Therapy* 4, 432-441] using the primers PRFse-F (5'- GAG GAG GAG GAG GAG GGC CGG CCG CGT CGA CCA GGT CAG AGT TGT GAG AGC ACT GGA TGC-3') (SEQ ID NO: 13) and PRAsc-B (5'- GAG GAG GAG GAG GGC GCG CCC CGT CGA CCC AGC AAT AAC TTC AGA CAT CAT TTC TGG-3') (SEQ ID NO: 14). The point-mutated mouse ER LBD 15 was amplified from pBabe/Myc-ER [Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* 23, 1686-1690] using the primers ERFse-F (5'- GAG GAG GAG GAG GAG GGC CGG CCG CCG AAA TGA AAT GGG TGC TTC AGG AGA C-3') (SEQ ID NO: 15) and ERAsc-B (5'- GAG GAG GAG GAG GAG GGC GCG CCC GAT CGT GTT 20 GGG GAA GCC CTC TGC TTC-3') (SEQ ID NO: 16). The resulting PCR products were then inserted into pcDNA3/E2C-VP16 [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* 95, 14628-14633], in between the E2C and VP16 coding regions, via digestion with the restriction endonucleases Fse1 and Asc1.

25 To generate fusion constructs with B3 or N1 DBDs, E2C was replaced by the B3 or N1 coding regions via Sfi1 digestion. Fusion constructs containing a VP64 effector domain were produced by replacing VP16 by the VP64 coding region via Asc1-Pac1 digestion.

Heterodimeric switch constructs. For construction of the E2C-ER 30 fusion, the point-mutated mouse ER LBD was amplified from pBabe/Myc-ER

[Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* **23**, 1686-1690] using the primers ERFse-F and ERPac-B (5'-GAG GAG GAG GAG GAG TTA ATT AAG ATC GTG TTG GGG AAG CCC TCT GCT TC-3') (SEQ ID NO: 17). The PCR product was then inserted 5 into the construct pcDNA3/E2C-VP64, replacing the VP64 coding region, via Fse1-Pac1 digestion. To generate the ER-VP64 fusion, the ER LBD was amplified using the primers ERATGBam-F (5'-GAG GAG GAG GAG GGA TCC GCC ACC ATG CGA AAT GAA ATG GGT GCT TCA GGA GAC-3') (SEQ ID NO: 18) and ERAsc-B. The PCR product was then inserted into 10 pcDNA3/E2C-VP64, [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633] replacing the E2C coding region, via BamH1-Asc1 digestion.

Single-chain switch constructs. For construction of single-chain fusions with two ER LBDs, the point-mutated mouse ER LBD was amplified from 15 pBabe/Myc-ER [Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* **23**, 1686-1690] either using the primers ERFse-F and ERSpe1-B (5'-GAG GAG GAG GAG GAG ACT AGT GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA GAT CGT GTT GGG GAA GCC CTC TGC-3') (SEQ ID NO: 19), or using the primers 20 ERNhe1-F1 (for 18aa linker construct; 5'-GAG GAG GAG GAG GAG GAG GCT AGC GGC GGT GGC GGT GGC TCC TCT GGT GGC GGT GGC GGT TCT TCC AAT GAA ATG GGT GCT TCA GGA GAC-3') (SEQ ID NO: 20) or ERNhe1-F2 (for 30aa linker construct; 5'- GAG GAG GAG GAG GAG GAG GCT AGC TCT TCC AAT GAA ATG GGT GCT TCA GGA GAC -3') (SEQ ID 25 NO: 21), and ERAsc-B. The PCR products were then digested with, respectively, Fse1 and Spe1, or Nhe1 and Asc1, and inserted into Fse1-Asc1 linearized pcDNA3/E2C-VP64 [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633].

For construction of RXR-EcR single-chain fusions, the ligand binding 30 domain of the human retinoid X receptor (hRXR α , aa373-654) was PCR amplified from pVgRXR (Invitrogen) using the primers RXRFse-F (5'-GAG

GAG GAG GGC CGG CCG GGA AGC CGT GCA GGA GGA GCG GC-3') (SEQ ID NO: 22) and RXRSpe-B (5'-GAG GAG GAG GAG GAG ACT AGT GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA AGT CAT TTG GTG CGG CGC CTC CAG C-3') (SEQ ID NO: 23). The ligand 5 binding domain of the ecdysone receptor (EcR, aa202-462, *drosophila melanogaster*) was PCR amplified from pVgRXR using the primers EcRNhe-F1 (for 18aa linker construct; 5'-GAG GAG GAG GAG GCT AGC TCT TCC GGT GGC GGC CAA GAC TTT GTT AAG AAG G-3') (SEQ ID NO: 24), or EcRNhe-F2 (for 30aa linker construct; 5'-GAG GAG GAG GAG GCT AGC 10 GGC GGT GGC GGT GGC TCC TCT GGT GGC GGT GGC GGT TCT TCC GGT GGC GGC CAA GAC TTT GTT AAG AAG G-3') (SEQ ID NO: 25), and EcRAsc-B (5'-GAG GAG GAG GGC GCG CCC GGC ATG AAC GTC CCA GAT CTC CTC GAG-3') (SEQ ID NO: 26). The PCR products were then digested with, respectively, Fse1 and Spe1, or Nhe1 and Asc1, and inserted into 15 Fse1-Asc1 linearized pcDNA3/E2C-VP64 [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* 95, 14628-14633]. DNA binding domains were exchanged via Sfi1 digestion, effector domains via Asc1-Pac1 digestion.

To generate the 36aa linker, E2C-RLLE-VP64 fusion construct, the RXR 20 LBD was PCR amplified from pcDNA3/E2C-RE-VP64 using the primers RXRFse-F and RXRSpeLL-B (5'-GAG GAG GAG GAG GAG ACT AGT AGA GCC ACC GCC CCC TTC AGA ACC GCC CGA GCC ACC GCC ACC AGA GG-3') (SEQ ID NO: 27). The EcR LBD was amplified from the same plasmid, using the primers EcRNheLL-F (5'-GAG GAG GAG GAG GCT AGC GGG 25 GGT TCG GAG GGT GGC GGG TCT GAG GGT GGG GGT GGT TCC ACT AGC TCT TCC-3') (SEQ ID NO: 28) and EcRAsc-B. The PCR products were inserted into pcDNA3/E2C-VP64 as described above.

EXAMPLE 2: Gene Switches

30 **Generation of hormone-regulated zinc finger-steroid receptor fusion proteins.** Previous studies have shown the potential of engineered C2-H2 zinc

finger proteins for the regulation of target gene expression [Liu, Q., Segal, D. J., Ghiara, J. B., and Barbas, C. F., III (1997) *Proc. Natl. Acad. Sci. USA* **94**, 5525-5530; Kim, J. S., and Pabo, C. O. (1997) *J Biol Chem* **272**, 29795-29800; Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633; Beerli, R. R., Dreier, B., and Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* **97**, 1495-1500]. However, to fully realize the potential of engineered zinc finger proteins, it is desirable that their otherwise constitutive DNA binding activity be rendered ligand-dependent. The ligand binding domains (LBDs) of the human progesterone receptor (hPR) and the murine estrogen receptor (mER) have previously been used for the regulation of heterologous proteins, after having been modified to lack binding to the natural hormones while retaining binding to synthetic antagonists [Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* **23**, 1686-1690; Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) *Gene Therapy* **4**, 432-441]. Thus, the Zif268 variant C7 [Wu, H., Yang, W.-P., and Barbas, C. F., III (1995) *Proc. Natl. Acad. Sci. USA* **92**, 344-348] was fused to a transcriptional activation domain plus the LBD of either of the two nuclear hormone receptors. The VP64-C7-PR fusion protein contains an N-terminal VP64 activation domain [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633], and a C-terminal hPR LBD (aa645-914) lacking amino acids 915-933, responsive to the progesterone-antagonist RU486/Mifepristone but not to progesterone [Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) *Gene Therapy* **4**, 432-441]. The VP64-C7-ER fusion protein contains a C-terminal mER LBD (aa282-599) with a single amino acid substitution (G525R), and is responsive to the estrogen antagonist 4-hydroxy-tamoxifen (4-OHT) but not to estrogen [Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* **23**, 1686-1690].

Determination of the optimal response element for zinc finger-steroid receptor fusion proteins. Naturally occurring steroid receptors bind DNA as

dimers and typically recognize response elements consisting of palindromic sequences [Evans, R. M. (1988) *Science* **240**, 889-895; Carson-Jurica, M. A., Schrader, W. T., and O'Malley, W. (1990) *Endocrine Reviews* **11**, 201-220]. Moreover, it was demonstrated that in some cases also direct repeats can serve as binding sites for receptor dimers [Aumais, J. P., Lee, H. S., DeGannes, C., Horsford, J., and White, J. H. (1996) *J. Biol. Chem.* **271**, 12568-12577]. Given this obvious flexibility in DNA recognition by naturally occurring receptor dimers, the optimal structure of a response element for an artificial, zinc finger based transcriptional switch was not known. However, to develop an efficient, hormone-inducible system for the regulation of target gene expression, a detailed knowledge of the binding site architecture is required.

To determine the optimal orientation and spacing of the two half-sites of a response element for a zinc finger-LBD fusion protein, a series of reporter plasmids was constructed. Each contains two C7 binding sites upstream of a TATA box and a firefly luciferase coding region. The two C7 binding sites were introduced in different orientations (direct, inverted, or everted repeat) and with various spacings (no spacing or 1 to 5 bp spacing). Plasmids directing expression of VP64-C-PR or VP64-C7-ER fusion constructs were then co-transfected with the various reporter plasmids and assayed for hormone-induced luciferase expression. Significantly, each of the C7 dimer binding sites was able to act as a response element for both PR and ER based proteins, albeit at varying efficiency. In contrast, a reporter plasmid with a single C7 binding site was not activated, indicating that hormone-induced activation of transcription was mediated by dimers.

Optimal spacing depended on the orientation of the two half-sites. In the case of the PR fusion protein, optimal spacing seemed to be at 2-3 bp for inverted repeats and 3 bp for everted repeats. Response elements consisting of direct repeats had no single optimal spacing; the best response was obtained with 4-5 bp, or no spacing at all. For the ER fusion protein, optimal spacing was at 3-4 bp for direct repeats, 1-2 bp for inverted repeats, and 3 bp for everted repeats. It should be noted that there were significant variations in the basal, i.e. ligand-independent

activity of PR and ER fusion proteins, depending on the response element tested. Most notably, increasing the spacing of direct repeats from 3 to 4 bp led to a 1.9-fold higher basal activity of VP64-C7-PR, and even a 3.7-fold increase in the case of VP64-C7-ER. High basal activity is extremely undesirable for an inducible promoter system, where tight control over the expression levels of a particular gene of interest is often required, especially if the gene product is toxic. Thus, in choosing appropriate response elements, particular attention must be paid not only to hormone inducibility but also to its basal activity in the presence of the regulatory protein. The response element consisting of direct repeats with a spacing of three nucleotides was considered to be a good choice for use in a hormone-inducible artificial promoter, since it was compatible with both PR and ER fusion proteins. Significantly, its basal activity in the presence of either PR or ER fusion proteins was among the lowest of all response elements tested. Furthermore, good hormone induced activation of transcription was observed with both VP64-C7-PR (3.9-fold) and VP64-C7-ER (9.5-fold).

Generation of novel DNA binding domains. While the use of the C7 DNA binding domain was well suited for the preliminary studies described above, it may not be a good choice for incorporation into an inducible transcriptional regulator. The C7 protein is a variant of the mouse transcription factor Zif268 [Pavletich, N. P., and Pabo, C. O. (1991) *Science* **252**, 809-817], with increased affinity but unchanged specificity [Wu, H., Yang, W.-P., and Barbas, C. F., III (1995) *Proc. Natl. Acad. Sci. USA* **92**, 344-348]. We reasoned that the use of alternate DNA binding domains would minimize potential pleiotropic effects of the chimeric regulators. Previously, we described a strategy for the rapid assembly of zinc finger proteins from a family of predefined zinc finger domains specific for each of the sixteen 5'-GNN-3' DNA triplets [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633; Segal, D. J., Dreier, B., Beerli, R. R., and Barbas, C. F., III (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2758-2763]. Three finger proteins binding any desired 5'-(GNN)₃-3' sequence can be rapidly prepared by grafting the amino acid residues involved in base-specific DNA recognition into the framework of the consensus

three finger protein Sp1C [Desjarlais, J. R., and Berg, J. M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2256-2260]. To date, well over 100 three finger proteins have been produced in our laboratory. Two of these, B3 and N1, were chosen to be used in inducible transcriptional regulators (Figure 1A). The B3 and N1 proteins 5 are designed to bind the sequences 5'-GGA GGG GAC-3' or 5'-GGG GTA GAA-3', respectively. To verify their DNA binding specificity, these proteins were purified as MBP-fusions and tested by ELISA analysis using an arbitrary selection of oligonucleotides containing 5'-(GNN)₃-3' sequences (Fig. 1B). Significantly, both proteins recognized their target sequence and showed no 10 crossreactivity to any of the other 5'-(GNN)₃-3' sequences tested. However, as judged by ELISA, binding of N1 was much weaker than binding of B3. Therefore, affinities were determined by electrophoretic mobility-shift analysis. The B3 protein bound its target sequence with a K_D value of 15nM, similar to the K_D values we previously reported for other three finger proteins [Beerli, R. R., 15 Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633]. In contrast, N1 affinity for its target was dramatically lower and we estimate its K_D value to be in the range of 5-10 μM. The fact that the two proteins had very different affinities for their respective target sequences was considered positive, since it allows to investigate the influence of affinity on the 20 functionality of an inducible expression system.

RU486- and 4-OHT-inducible systems for the control of gene expression. To allow for a comparative analysis, a series of RU486- or 4-OHT-inducible transcriptional regulators were constructed containing either the B3 or the N1 DNA binding domain. The role of placement of the activation domain was 25 investigated, by fusing it either to the N- or the C-terminus of the protein. Two different activation domains were compared: the *herpes simplex* virus VP16 transactivation domain [Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988) *Nature* **335**, 563-564], and the synthetic VP64 activation domain, which consists of 4 tandem repeats of VP16's minimal activation domain [Beerli, R. R., 30 Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633].

Synthetic promoters were constructed based on the B3 and N1 DNA target sequences, and the optimal response element structure defined above. The 10xB3-TATA-luc and 10xN1-TATA-luc plasmids each contain five response elements, consisting of direct repeats spaced by three nucleotides, upstream of a TATA box
 5 and a firefly luciferase coding region. The response elements are separated from each other by six nucleotides, which should allow the concomitant binding of five dimers and thus maximize the promoter activity. The activity of the various fusion constructs was assessed by transient cotransfection studies with the cognate TATA reporter plasmids in HeLa cells (Table 1).

10

Table 1

	LBD=PR		LBD=ER	
	exp. 1	exp. 2	exp. 1	exp. 2
VP16-B3-LBD	34x	36x	37x	26x
VP64-B3-LBD	37x	24x	26x	27x
B3-LBD-VP16	115x	116x	47x	58x
B3-LBD-VP64	110x	85x	62x	99x
VP16-N1-LBD	188x	159x	101x	39x
VP64-N1-LBD	206x	390x	49x	58x
N1-LBD-VP16	282x	203x	24x	30x
N1-LBD-VP64	151x	129x	1319x	464x

In general, the ER fusion proteins turned out to be the stronger
 15 transactivators, and 4-OHT-induced luciferase activity was usually 3 to 6 times higher than RU486-induced luciferase activity mediated by PR fusion proteins. However, since the basal, *i. e.* ligand independent, activity of ER chimeras was often somewhat higher, their hormone-induced fold-stimulation was not generally better. Hormone-dependent gene activation in excess of 2 orders of magnitude
 20 was commonly observed with both PR and ER fusion proteins, values that are significantly better than what was previously reported for the Gal4-PR fusion

protein GLVPC' [Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) *Gene Therapy* **4**, 432-441].

The placement of the activation domain had a significant influence on the activity of the chimeric regulators. However, favored placement was dependent
5 on the nature of the activation domain. Whereas the VP16 domain yielded the more potent activators when placed at the C-terminus, the VP64 was more active at the N-terminus. Accordingly, direct comparisons showed that an N-terminal VP64 was more potent than a N-terminal VP16 domain, and a C-terminal VP16 was more potent than a C-terminal VP64 domain. The nature and placement of
10 the activation domain was also found to have an influence on the basal activity of the chimeric regulators. In particular, a relatively high basal activity was observed in the case of regulators with N-terminal VP64 domain.

The nature of the DNA binding domain had a major influence on the extent of ligand-dependence of the chimeras. Use of the N1 protein as DNA
15 binding domain led to more tightly regulated fusion constructs with significantly better fold-stimulation of promoter activities than the use of B3, likely due to the dramatic affinity differences between N1 and B3. In particular, the N1-ER-VP64 regulator had no significant basal activity and was capable of mediating a 464- to 1319-fold 4-OHT-induced activation of the 10xN1-TATA minimal promoter
20 (Table 1). The extent of ligand-induced activation of gene expression over a range of 3 orders of magnitude is particularly remarkable, since it has thus far only been reported for the tetracycline controlled system of gene regulation [Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5547-5551; Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W., and Bujard, H. (1995) *Science* **268**, 1766-1769].

Concomitant regulation of multiple promoters. Zinc finger technology has made a large repertoire of DNA binding specificities available for use in protein engineering [Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14628-14633; Segal, D. J., Dreier, B., Beerli,
30 R. R., and Barbas, C. F., III (1999) *Proc. Natl. Acad.*

Sci. USA 96, 2758-2763; Beerli, R. R., Dreier, B., and Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* 97, 1495-1500]. The availability of different steroid hormone receptor-derived regulatory domains [Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G., and Evan, G. I. (1995) *Nucl. Acids Res.* 23, 1686-5 1690; Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) *Gene Therapy* 4, 432-441], and the ability to redirect chimeric regulators to virtually any desired target sequence should make it possible to independently regulate the expression of multiple genes at the same time. To examine this possibility, a reporter plasmid was constructed directing expression of β -galactosidase (β -gal) 10 under the control of the 10xN1-TATA minimal promoter. The chimeric regulators B3-PR-VP16 and N1-ER-VP64 were then transiently expressed in HeLa cells along with the 10xB3-TATA-luc and 10xN1-TATA- β -gal reporter plasmids. The transfected cells were treated with either RU486 or 4-OHT and the luciferase and β -gal activities were monitored. Significantly, RU486 induced expression of 15 luciferase while having no effect on β -gal reporter gene activity. 4-OHT, on the other hand, did not affect luciferase expression but efficiently activated β -gal expression. These results demonstrate that the two regulator/promoter combinations act independently from one another, and that multiple genes can efficiently and independently regulated by the selective addition of the desired 20 hormone.

Development of a monomeric hormone-dependent gene-switch. The ability to engineer DNA binding proteins with desired specificities makes it possible to generate artificial transcription factors capable of imposing dominant regulatory effects on endogenous genes [Beerli, R. R., Dreier, B., and Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* 97, 1495-1500]. For many applications of this technology it may be desirable that the effect on endogenous gene expression is reversible. The use of steroid hormone receptor LBDs has the potential to render regulation of endogenous gene expression reversible. However, one major drawback is the fact that steroid hormone receptors, as well as the chimeric 25 regulators described herein, bind DNA as dimers. Thus, when the fusion protein 30 regulators described herein, bind DNA as dimers. Thus, when the fusion protein

C7-ER-VP64 was transiently expressed in HeLa cells it was unable to regulate a reporter construct carrying a single C7 binding site, while it readily regulated a reporter that had two C7 binding sites and therefore accommodated binding of a dimer (Fig. 2B). An additional problem was encountered when the C7 DBD was
5 replaced by E2C, which contains six zinc finger domains and recognizes the 18-
bp sequence 5'-GGG GCC GGA GCC GCA GTG-3' (SEQ ID NO; 29) in the 5'-
UTR of the proto-oncogene *c-erbB-2* [Yamamoto, T., Ikawa, S., Akiyama, T.,
Semba, K., Nomura, N., Miyajima, N., Saito, T., and Toyoshima, K. (1986)
Nature 319, 230-234; Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F., III
10 (1998) *Proc. Natl. Acad. Sci. USA* 95, 14628-14633]. The E2C-ER-VP64 fusion
protein was constitutively active on a reporter carrying a single E2C binding site,
almost as active as an E2C-VP64 fusion without an ER LBD, and did not respond
well to hormone. Apparently, the use of a large DNA binding domain recognizing
an extended stretch of DNA with high affinity renders the chimera hormone- and
15 dimerization-independent.

To overcome these problems, we produced two types of ER-based
chimeric regulators, designed to be capable of regulating gene expression through
a single binding site in a hormone-dependent manner. In the first strategy, a
heterodimeric regulator was generated consisting of the engineered zinc finger
20 protein E2C fused to an ER LBD, as well as an ER LBD fused to a VP64
activation domain (Fig. 2A). When this heterodimeric regulator was expressed in
HeLa cells, it had no significant activity on the E2C-TATA-luc reporter plasmid
in the absence of 4-OHT. Addition of hormone led to a 3- to 5-fold stimulation of
luciferase expression, indicating the formation of functional heterodimers.
25 However, hormone-induced reporter gene activation was significantly lower than
that induced by an E2C-VP64 fusion protein, presumably at least in part due to
the formation of E2C-ER and ER-VP64 homodimers. Homodimers were inactive,
since neither E2C-ER nor ER-VP64 alone induced luciferase expression. In the
second strategy, fusion proteins were generated by combining the dimerization
30 partners E2C-ER and ER-VP64 in one single polypeptide, through a flexible
polypeptide linker. Two linkers were tested, 18 and 30 amino acids in length,

creating the proteins E2C-scER/18-VP64 and E2C-scER/30-VP64 (Fig. 2A). These proteins were expected to be activated via intramolecular, rather than intermolecular, dimerization and therefore functional as monomers. Combination of two ER LBDs into one single-chain fusion construct should allow a more 5 efficient hormone-induced dimerization and therefore yield more efficient activators. Indeed, when E2C-scER/18-VP64 and E2C-scER/30-VP64 were transiently expressed in HeLa cells, they efficiently activated the E2C-TATA-luc reporter in a largely hormone-dependent manner (Fig. 2B, 2C and 2D). Thus, dimeric regulators requiring response elements similar to those of natural steroid 10 hormone receptors were successfully converted into monomeric, ligand-dependent transcription factors.

Monomeric gene-switch based on EcR and RXR LBDs. To show that the production of a ligand-dependent monomeric gene switch by fusion with two LBDs is a generally applicable strategy, the utility of other nuclear hormone 15 receptors was tested. In particular, utility of the LBDs of the *Drosophila* ecdysone receptor (EcR) was investigated. In *Drosophila*, this receptor functions as a heterodimer between EcR and the product of the *ultraspiracle* (USP) gene [Yao, T.-P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P., and Evans, R. M. (1993) *Nature* **366**, 476-479]. However, it has been shown 20 that EcR also efficiently heterodimerizes with USP's vertebrate homologue retinoid X receptor (RXR) in response to the ecdysone agonists Muristerone A or Ponasterone A (PonA) [Nakanishi, K. (1992) *Steroids* **57**, 649-657; Yao, T.-P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P., and Evans, R. M. (1993) *Nature* **366**, 476-479; No, D., Yao, T.-P., 25 and Evans, R. M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 3346-3351]. The EcR and RXR LBDs were therefore used to prepare a monomeric gene switch analogous to the scER chimeras described above (Fig. 3A). Thus, the human RXR α LBD (aa373-654) and the *Drosophila* EcR LBD (aa202-462) were inserted in between 30 the E2C DBD and the VP64 activation domain, creating E2C-RE-VP64. In this fusion construct, the two LBDs are connected by an 18 amino acid flexible linker, the same that was used in E2C-scER/18-VP64. When this chimeric regulator was

transiently expressed in HeLa cells along with the E2C-TATA-luc reporter plasmid, significant basal activity was observed. However, activity could be increased 3-fold by PonA, showing that this artificial construct was hormone-responsive. To improve the ligand dependence, the length of the linker connecting 5 the RXR and EcR LBDs was increased, a measure that seemed beneficial in the case of the single-chain ER constructs. A longer linker should allow the LBDs to optimize their contact and add to the conformational disorder in the unliganded state. Indeed, when the linker was elongated to 30 aa (in E2C-RLE-VP64) or 36 aa (in E2C-RLLE-VP64), basal activity was significantly reduced and PonA led 10 to a 9- to 10-fold activation, an extent of responsiveness comparable to the one of the single-chain ER fusion constructs (Fig. 3B). Thus, serial connection of pairs of nuclear hormone receptor LBDs appears to be a generally applicable strategy to render monomeric DNA binding proteins ligand-dependent.

The hPR and mER LBDs used for the fusion proteins did not encompass 15 their natural SV40-like nuclear localization signals (NLS), located between amino acids 637 and 644 in hPR, and between amino acids 260 and 267 in mER [Carson-Jurica, M. A., Schrader, W. T., and O'Malley, W. (1990) *Endocrine Reviews* 11, 201-220]. While it has been shown that this NLS is not required for 20 hormone-dependent nuclear localization of hPR, regulation of the subcellular localization of steroid receptors appears to be complex, and it was not *a priori* clear whether the presence of the SV40-like NLS was required for proper function of the chimeric proteins. Thus, additional constructs were prepared that incorporated an SV40 NLS (PKKKRKV) (SEQ ID NO: 30) in single letter amino acid code), either between VP16 and C7, or between C7 and LBD.

25 The chimeric transcriptional regulators were then tested for their ability to regulate the 10xC7-TATA-luc reporter plasmid in a hormone dependent manner. 10xC7-TATA-luc contains ten C7 binding sites [5'-GCG TGG GCG-3'] spaced by 5 nucleotides, and a TATA box, upstream of the firefly luciferase coding region. Each of the fusion proteins upregulated expression of luciferase in a largely 30 hormone dependent manner. RU486 stimulated the activity of VP16-C7-PR 26-fold, while 4-OHT led to a 43-fold activation of VP16-C7-ER. There was no

detectable crossreactivity between RU486 and ER, or between 4-OHT and PR. The presence of a NLS in either position was not only not required, but even undesirable, since it led to an increased basal (*i.e.* hormone-independent) activity of the fusion constructs, presumably through increased nuclear localization. Thus,
5 the hPR (aa645-914) and mER (aa281-599, G525R) LBDs are able to confer hormone-dependence onto the zinc finger protein C7.

The ability to reversibly control the expression of multiple genes, or alleles of a gene, could prove very useful for many basic research applications. In particular, selective and independent expression of one gene, but not another (and
10 *vice versa*), by small and nontoxic ligands would allow for a comparative analysis of gene function, both *in vitro* and *in vivo*. We have shown that our modular system for controlling target gene expression is indeed able to independently control the expression of two genes within the same transfected cell, as evidenced by RU486-dependent luciferase induction and 4-OHT-induced β-gal expression.
15 The lack of β-gal induction by RU486, and luciferase induction by 4-OHT convincingly demonstrates the specificity of the chimeric regulators described here. Not only is the exquisite specificity of the utilized DNA binding domains retained, but also there is no detectable crossreaction between RU486 and the ER LBD, or between 4-OHT and the PR LBD.

20

WHAT IS CLAIMED IS:

1. A non-naturally occurring polypeptide comprising two ligand binding domains derived from nuclear hormone receptors operatively linked to a first functional domain.
5
2. The polypeptide of claim 1 wherein the two ligand binding domains are covalently linked by means of a peptide linker.
- 10 3. The polypeptide of claim 2 wherein the linker contains from about 10 to about 40 amino acid residues.
- 15 4. The polypeptide of claim 2 wherein the linker contains from about 15 to about 35 amino acid residues.
5. The polypeptide of claim 2 wherein the linker contains from about 18 to about 30 amino acid residues.
15
- 20 6. The polypeptide of claim 1 wherein the first and second ligand binding domains are derived from different nuclear hormone receptors.
7. The polypeptide of claim 1 wherein the first and second binding domains are derived from the same nuclear hormone receptor.
25
8. The polypeptide of claim 1 wherein the nuclear hormone receptor is an estrogen receptor, a progesterone receptor, an ecdysone receptor or a retinoid X acid receptor.
30
9. The polypeptide of claim 7 wherein at least one of the ligand binding domains is derived from a retinoid X acid receptor.

10. The polypeptide of claim 1 wherein the first functional domain is a DNA binding domain.

11. The polypeptide of claim 10 wherein the DNA binding domain
5 comprises at least one zinc finger DNA binding motif.

12. The polypeptide of claim 11 that comprises from two to twelve zinc finger DNA binding motifs.

10 13. The polypeptide of claim 11 that comprises from two to six zinc finger binding motifs.

14. The polypeptide of claim 11 wherein the zinc finger DNA binding motifs specifically bind to a nucleotide sequence of the formula (GNN)₁₋₆, where
15 G is guanidine and N is any nucleotide.

15. The polypeptide of claim 1 wherein the first functional domain is a transcriptional regulating domain.

20 16. The polypeptide of claim 1 further comprising a second functional domain operatively linked to either one of the ligand binding domains or the first functional domain.

25 17. The polypeptide of claim 16 wherein the first functional domain is a DNA binding domain and the second functional domain is a transcriptional regulating domain.

18. The polypeptide of claim 17 wherein the DNA binding domain comprises at least one zinc finger DNA binding motif.

30

19. The polypeptide of claim 18 that comprises from two to twelve

zinc finger DNA binding motifs.

20. The polypeptide of claim 18 that comprises from two to six zinc finger DNA binding motifs.

5

21. The polypeptide of claim 18 wherein the zinc finger DNA binding motifs specifically bind to a nucleotide sequence of the formula (GNN)₁₋₆, where G is guanidine and N is any nucleotide.

10 22. The polypeptide of claim 17 wherein the transcriptional regulating domain is an activation domain.

23. The polypeptide of claim 17 wherein the transcriptional regulating domain is a repression domain.

15

24. A non-naturally occurring polypeptide comprising (a) a DNA binding domain having from two to six zinc finger DNA binding motifs; (b) a first ligand binding domain derived from a retinoid X receptor operatively linked to the DNA binding domain, a second ligand binding domain derived from an ecdyzone receptor operatively linked to the first ligand binding domain with a peptide spacer of from 18 to 36 amino acid residues; and (c) a transcriptional regulating domain operatively linked to the second ligand binding domain.

25 20 25. A non-naturally occurring polypeptide comprising (a) a DNA binding domain having from three to six zinc finger DNA binding motifs; (b) a first ligand binding domain derived from a progesterone receptor operatively linked to the DNA binding domain, a second ligand binding domain derived from a progesterone receptor linked to the first ligand binding domain with a peptide spacer of from 18 to 36 amino acid residues; and (c) a transcriptional regulating 30 domain operatively linked to the second ligand binding domain.

26. A polynucleotide that encodes the polypeptide of claim 1.
27. A polynucleotide that encodes the polypeptide of claim 17.
- 5 28. An expression vector comprising the polynucleotide of claim 26.
29. An expression vector comprising the polynucleotide of claim 27.
30. A cell containing the polynucleotide of claim 26.
- 10 31. A cell containing the polynucleotide of claim 27.
32. A host cell transformed with the expression vector of claim 28.
- 15 33. A host cell transformed with the expression vector of claim 29.
34. A process of regulating the function of a target nucleotide that contains a defined sequence, the process comprising exposing the target nucleotide to the polypeptide of claim 1 in the presence of a ligand that binds one of the ligand binding domains of the polypeptide, wherein the DNA binding domain of the polypeptide binds the defined sequence.
- 20
35. A process of regulating the function of a target nucleotide that contains a defined sequence, the process comprising exposing the target nucleotide to the polypeptide of claim 17 in the presence of a ligand that binds one of the ligand binding domain of the polypeptide, wherein the functional domain of the polypeptide alters the function of the target nucleotide.
- 25

B3	<u>antiparallel β sheet</u>	<u>α helix</u>
F1 (GAC)	AQAALEPKEKPYACPECGKSFSDPGNLIVRHQRTHTGEK	
F2 (GGG)		PYKCPECGKSFSSRSDFKLIVRHQRTHTGEK
F3 (GGA)		PYKCPECGKSFSSQSSHLIVRHQRTHTGKKTSGQAG
 N1	<u>antiparallel β sheet</u>	<u>α helix</u>
F1 (GTA)	AQAALEPKEKPYACPECGKSFSSQSSSIIVRHQRTHTGEK	
F2 (GAA)		PYKCPECGKSFSSQSSNLIVRHQRTHTGEK
F3 (GGG)		PYKCPECGKSFSSRSDFKLIVRHQRTHTGKKTSGQAG

FIG. 1A

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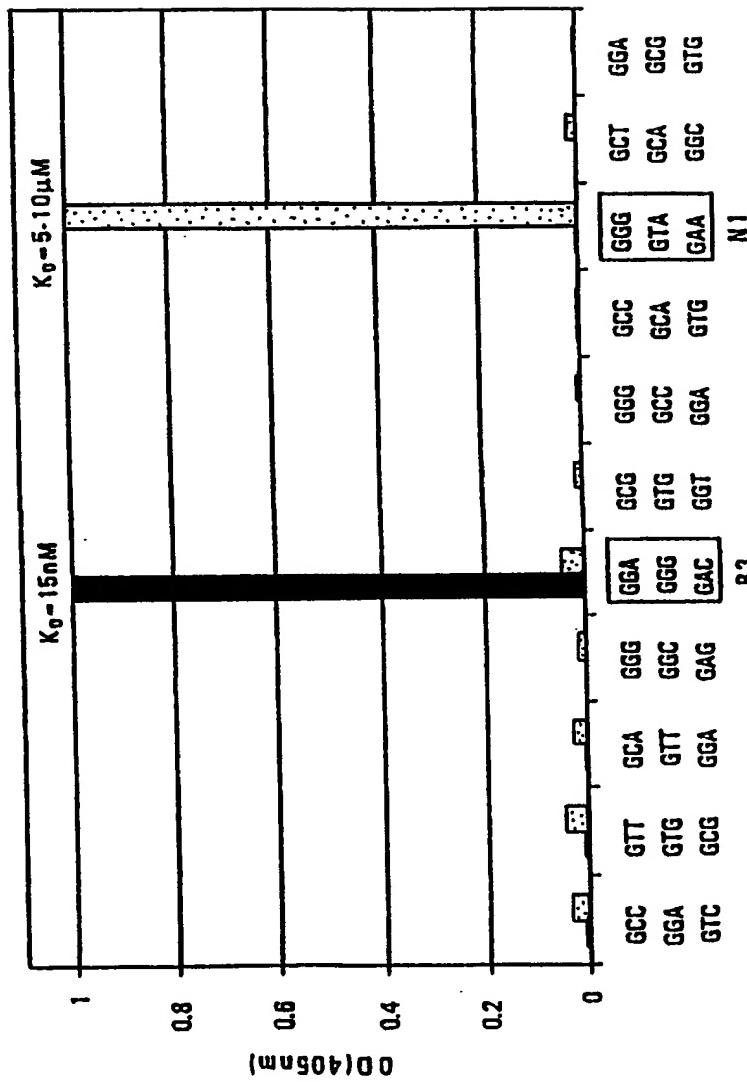
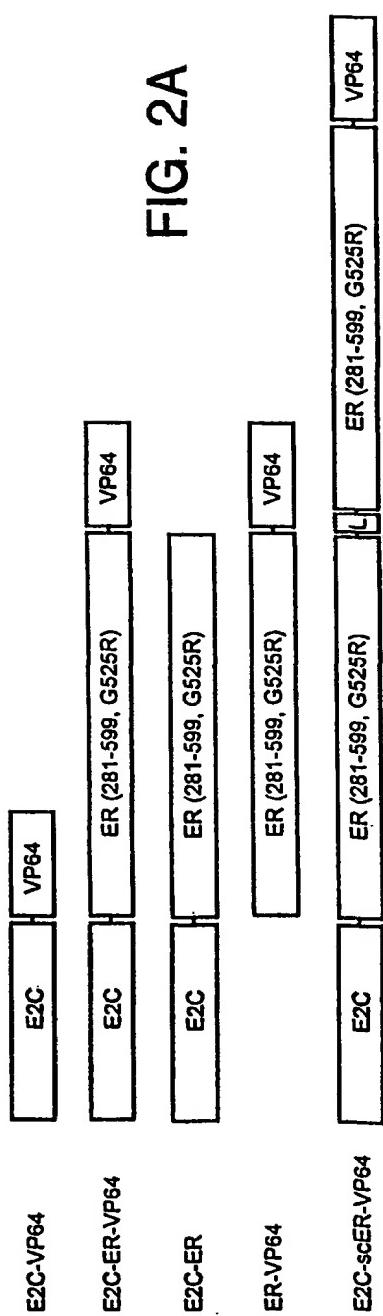
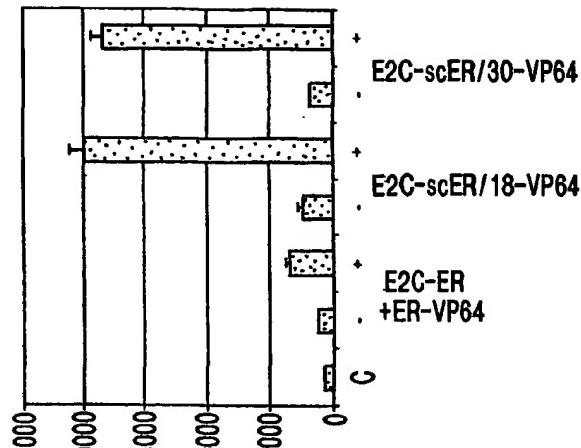
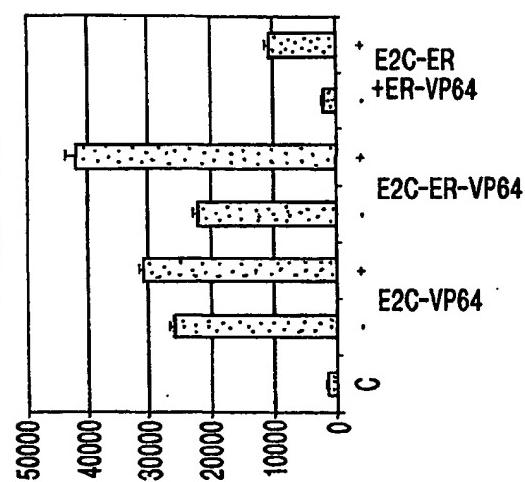
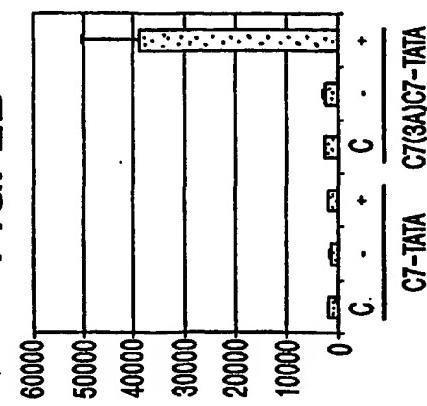


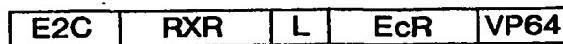
FIG. 1B

**FIG. 2D****FIG. 2C****FIG. 2B**

4 / 16



$L = 18 \text{ aa}$ E2C-RE-VP64



$L = 30 \text{ aa}$ E2C-RLE-VP64



$L = 36 \text{ aa}$ E2C-RLLE-VP64

FIG. 3A

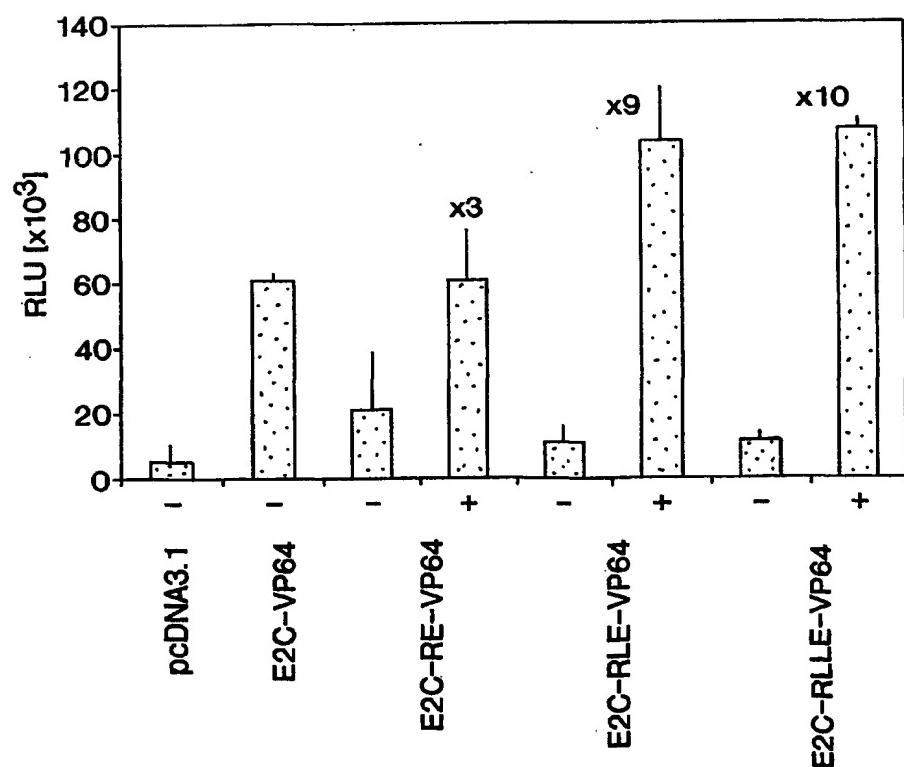


FIG. 3B

5/16

10	20	30	40	50	60
GCC CAG GCG GCC	CTC GAG CCC GGG GAG AAG CCC TAT GCT TGT CCG GAA TGT GGT AAG				
CGG GTC CGC CGG	GAG CTC GGG CCC CTC TTC GGG ATA CGA ACA GGC CTT ACA CCA TTC				
X A Q A A	L E P G B K P Y A C P E C G K>				
70	80	90	100	110	120
TCC TTC AGT CGC AGC GAT GTG CTG GTG CGC CAC CAG CGT ACC CAC ACG GGT GAA AAA CCG					
AGG AAG TCA GCG TCG CTA CAC GAC CAC GCG GTG GTC GCA TGG GTG TGC CCA CTT TTT GGC					
S F S R S D V L V R H Q R T H T G E B K P>					
130	140	150	160	170	180
TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGC CGC AGC GAT GAT CTG GTT CGC CAT CAA					
ATA TTT ACG GGT CTC ACG CCG TTT AGA AAA TCG GCG TCG CTA CTA GAC CAA GCG GTA GTT					
Y K C P E C G K S F S R S D D L V R H Q>					
190	200	210	220	230	240
CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA GAA TGT GGC AAG TCT TTC TCC CAG					
GCG TGA GTA TGA CCG CTC TTC GGT ATG TTT ACA GGT CTT ACA CGC TTC AGA AAG AGG GTC					
R T H T G E K P Y K C P E C G K S F S Q>					
250	260	270	280	290	300
TCT AGC CAC CTG GTT CGC CAC CAA CGT ACT CAC ACC GGG GAG AAG CCC TAT GCT TGT CCG					
AGA TCG GTG GAC CAA GCG GTG GTT GCA TGA GTG TGG CCC CTC TTC GGG ATA CGA ACA GGC					
S S H L V R H Q R T H T G E K P Y A C P>					
310	320	330	340	350	360
GAA TGT GGT AAG TCC TTC AGC CGC AGC GAT AAC CTG GTG CGC CAC CAG CGT ACC CAC ACG					
CTT ACA CCA TTC AGG AAG TCG GCG TCG CTA TTG GAC CAC GCG GTG GTC GCA TGG GTG TGC					
E C G K S F S R S D N L V R H Q R T H T>					
370	380	390	400	410	420
GGT GAA AAA CCG TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGC CAG GCC GGC CAC CTG					
CCA CTT TTT GGC ATA TTT ACG GGT CTC ACG CCG TTT AGA AAA TCG GTC CGG CCG GTG GAC					
G E K P Y K C P E C G K S F S Q A G H L>					
430	440	450	460	470	480
GCC AGC CAT CAA CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA GAA TGT GGC AAG					
CGG TCG GTA GTT GCG TGA GTA TGA CCG CTC TTC GGT ATG TTT ACA GGT CTT ACA CGC TTC					
A S H Q R T H T G E K P Y K C P E C G K>					
490	500	510	520	530	540
TCT TTC AGT GAT TGT CGT GAT CTT GCG AGG CAC CAA CGT ACT CAC ACC GGT AAA AAA ACT					
AGA AAG TCA CTA ACA GCA CTA GAA CGC TCC GTG GTT GCA TGA GTG TGG CCA TTT TTT TGA					
S F S D C R D L A R H Q R T H T G K K T>					
550					
AGT [GGC CAG GCC GGC CNN					
TCA [CCG GTC CGG CGG CNN					
S G Q A G X>					

FIG. 4

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10	20	30	40	50	60
NNG GCC CAG GCG GGC CTC GAG CCC TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT NNC CGG GTC CGC CGG GAG CTC GGG ATA CGA ACG GGA CAG CTC AGG ACG CTA CGG GCG AAA					
X	A	Q	A	A	L E P Y A C P V E S C D R R F>
70	80	90	100	110	120
TCT AAG TCG GCT GAT CTG AAG CGC CAT ATC CGC ATC CAC ACA GCA GGC CAG AAG CCC TTC CAG AGA TTC AGC CGA CTA GAC TTC GCG GTA TAG GCG TAG GTG TGT CGG GTC TTC GGG AAG GTC					
S	K	S	A	D L K R H I R I H T G Q K P F Q>	
130	140	150	160	170	180
TGT CGA ATA TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC ACA GCT TAT ACG TAC GCA TTG AAG TCA GCA TCA CTG GTG GAA TGG TGG GTG TAG GCG TGG					
C	R	I	C	M R N F S R S D H L T T H I R T>	
190	200	210	220	230	240
CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GTG TGT CCG CTC TTC GGA AAA CGG ACA CTG TAA ACA CCC TCC TTC AAA CGG TCC TCA CTA					
H	T	G	E	K P F A C D I C G R K F A R S D>	
250	260	270	280	290	300
GAA CGC AAG AGG CAT ACC AAA ATC CAT ACC GGT GAG AAG CCC TAT GCT TGC CCT GTC GAG CTT GCG TTC TCC GTA TGG TTT TAG GTA TGG CCA CTC TTC GGG ATA CGA ACG GGA CAG CTC					
E	R	K	R	H T K I H T G E K P Y A C P V E>	
310	320	330	340	350	360
TCC TGC GAT CGC CGC TTT TCT AAG TCG GCT GAT CTG AAG CGC CAT ATC CGC ATC CAC ACA AGG ACG CTA CGC GCG AAA AGA TTC AGC CGA CTA GAC TTC GCG GTA TAG GCG TAG GTG TGT					
S	C	D	R	R F S K S A D L K R H I R I H T>	
370	380	390	400	410	420
GGC CAG AAG CCC TTC CAG TGT CGA ATA TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CCT CCG GTC TTC GGG AAG GTC ACA GCT TAT ACG TAC GCA TTG AAG TCA GCA TCA CTG GTG GAA					
G	Q	K	P	F Q C R I C M R N F S R S D H L>	
430	440	450	460	470	480
ACC ACC CAC ATC CGC ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG TGG TGG GTG TAG GCG TGG GTG TGT CCG CTC TTC GGA AAA CGG ACA CTG TAA ACA CCC TCC					
T	T	H	I	R T H T G E K P F A C D I C G R>	
490	500	510	520	530	540
AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG GAC TTC AAA CGG TCC TCA CTA CTT GCG TTC TCC GTA TGG TTT TAG GTA AAT TCT GTC TTC CTG					
K	F	A	R	S D E R K R H T K I H L R Q K D>	
550	560				
TCT AGA ACT AGT GGC CAG GCC GGC CNN AGA TCT TGA TCA CCG GTC CGG CGG CNN					
S	R	T	S	G Q A G X>	

FIG. 5

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10	20	30	40	50	60
NNG GCC CAG GCG GCC	CTC GAG CCC GGG GAG AAG CCC TAT GCT TGT CCG GAA TGT GGT AAG				
NNC CGG GTC CGC CGG	GAG CTC GGG CCC CTC TTC GGG ATA CGA ACA GGC CTT ACA CCA TTC				
X A Q A A L E P G E K P Y A C P E C G K>					
70	80	90	100	110	120
TCC TTC AGC ACC AGT GGC CAC CTG GTG CGC CAC CAG CGT ACC CAC ACG GGT GAA AAA CCG					
AGG AAG TCG TGG TCA CCG GTG GAC CAC GCG GTG GTC GCA TGG GTG TGC CCA CTT TTT GGC					
S F S T S G H L V R H Q R T H T G E K P>					
130	140	150	160	170	180
TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGT CGC AGC GAT GTG CTG GTG CGC CAT CAA					
ATA TTT ACG GGT CTC ACG CCG TTT AGA AAA TCA GCG TCG CTA CAC GAC CAC GCG GTA GTT					
Y K C P E C G K S F S R S D V L V R H Q>					
190	200	210	220	230	240
CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA GAA TGT GGC AAG TCT TTC TCA CGT					
GCG TGA GTA TGA CCG CTC TTC GGT ATG TTT ACA GGT CTT ACA CCG TTC AGA AAG AGT GCA					
R T H T G E K P Y K C P E C G K S F S R>					
250	260	270	280	290	300
TCA GAC GAC TTG GTC CGT CAC CAA CGT ACT CAC ACC GGG GAG AAG CCC TAT GCT TGT CCG					
AGT CTG CTG AAC CAG GCA GTG GTT GCA TGA GTG TGG CCC CTC TTC GGG ATA CGA ACA GGC					
S D D L V R H Q R T H T G E K P Y A C P>					
310	320	330	340	350	360
GAA TGT GGT AAG TCC TTC AGT GAT CCT GGC AAC CTG GTT CGC CAC CAG CGT ACC CAC ACG					
CTT ACA CCA TTC AGG AAG TCA CTA GGA CCG TTG GAC CAA GCG GTG GTC GCA TGG GTG TGC					
E C G K S F S D P G N L V R H Q R T H T>					
370	380	390	400	410	420
GGT GAA AAA CCG TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGT CGC TCC GAT AAA CTG					
CCA CTT TTT GGC ATA TTT ACG GGT CTC ACG CCG TTT AGA AAA TCA GCG AGG CTA TTT GAC					
G E K P Y K C P E C G K S F S R S D K L>					
430	440	450	460	470	480
GTG CGC CAT CAA CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA GAA TGT GGC AAG					
CAC GCG GTA GTT GCG TGA GTA TGA CCG CTC TTC GGT ATG TTT ACA GGT CTT ACA CCG TTC					
V R H Q R T H T G E K P Y K C P E C G K>					
490	500	510	520	530	540
TCT TTC TCC CAG TCT AGC CAC CTG GTT CGC CAC CAA CGT ACT CAC ACC GGT AAA AAA ACT					
AGA AAG AGG GTC AGA TCG GTG GAC CAA GCG GTG GTT GCA TGA GTG TGG CCA TTT TTT TGA					
S F S Q S S H L V R H Q R T H T G K K T>					
550					
AGT GGC CAG GCC GGC GNN					
TCA CCG GTC CGG CCG GNN					
S G Q A G X>					

FIG. 6

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GGGCGCGCCCAGGAAACTATGACCTTCAAAGACGTTGAAGTTACCTTCTCTCAGGACGAATGGGGTTGGC
TGGACTCCGCTCAGCGTAACCTGTACCGTGACGTTATGCTGGAAAACCTACCGCAACATGGCTTCCCTGGT
TGGCGGCGGCCGCGGTGGTCAGGAAACTATGACCTTCAAAGACGTTGAAGTTACCTTCTCTCAGGACGAA
TGGGGTTGGCTGGACTCCGCTCAGCGTAACCTGTACCGTGACGTTATGCTGGAAAACCTACCGCAACATGG
CTTCCCTGGTTGGCTTAATTAAAC

FIG. 7

GGGCGCGCCGCTGCCGTGCGCATGAACATCCAGATGCTGCTCGAACGCCGCTGATTATCTGGAACGCCGGG
ACCGCGAAGCCGAGCACGGCTACGCCAGCATGCTGCCATATGGCGGCCGCGGTGGTGCCTGCCGTGCG
CATGAACATCCAGATGCTGCTCGAACGCCGCTGATTATCTGGAACGCCGGGAGCCGCGAACGCCGAGCACGGC
TACGCCAGCATGCTGCCATATTTAATTAAAC

FIG. 8

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10 20 30 40 50 60
 GGA TCC GCC ACC ATG GCC CAG GCG CCC GTC GAG CCC GGG GAG AAG CCC TAT GCT TGT CCG
 CCT AGG CGG TGG TAC CGG GTC CGC CGG GAG CTC GGG CCC CTC TTC GGG ATA CGA ACA GGC
 G S A T M A Q A A L E P G E K P Y A C P>

 70 80 90 100 110 120
 GAA TGT GGT RAG TCC TTC AGT AGG AAG GAT TCG CTT GTG AGG CAC CAG CGT ACC CAC ACG
 CTT ACA CCA TTC AGG AAG TCA TCC CTA AGC GAA CAC TCC GTG GTC GCA TGG GTG TGC
 E C G K S F S R K D S L V R H Q R T H T>

 130 140 150 160 170 180
 GGT GAA AAA CCG TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGT CAG TCG GGG GAT CTT
 CCA CTT TTT GGC ATA TTT ACG GGT CTC ACG CCG TTT AGA AAA TCA GTC AGC CCC CTA GAA
 G E K P Y K C P E C G K S F S Q S G D L>

 190 200 210 220 230 240
 AGG CGT CAT CAA CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA GAA TGT GGC AAG
 TCC GCA GTA GTT GCG TGA TGA CCG CTC TTC GGT ATG TTT ACA GGT CTT ACA CGG TTC
 R R H Q R T H T G E K P Y K C P E C G K D>

 250 260 270 280 290 300
 TCT TTC AGT GAT TGT CGT GAT CTT GCG AGG CAC CAA CGT ACT CAC ACC GGG GAG AAG CCC
 AGA AAG TCA CTA ACA GCA CTA GAA CGC TCC GTG GTT GCA TGA GTG TGG CCC CTC TTC GGG
 S F S D C R D L A R H Q R T H T G E K P>

 310 320 330 340 350 360
 TAT GCT TGT CCG GAA TGT GGT AAG TCC TTC TCT CAG AGC TCT CAC CTG GTG CGC CAC CAG
 ATA CGA ACA GGC CTT ACA CCA TTC AGG AAG AGA GTC TCG AGA GTG GAC CAC GCG GTG GTC
 Y A C P E C G K S F S Q S S H L V R H Q>

 370 380 390 400 410 420
 CGT ACC CAC ACG GGT GAA AAA CCG TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGT GAC
 GCA TGG GTG TGC CCA CTT TTT GGC ATA TTT ACG GGT CTC ACG CCG TTT AGA AAA TCA CTG
 R T H T G E K P Y K C P E C G K S F S D>

 430 440 450 460 470 480
 TGC CGC GAC CTT GCT CGC CAT CAA CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA
 ACG GCG CTG GAA CGA GCG GTA GTT GCG TGA GTA TGA CCG CTC TTC GGT ATG TTT ACA GGT
 C R D L A R H Q R T H T G E K P Y K C P>

 490 500 510 520 530 540
 GAA TGT GGC AAG TCT TTC AGC CGC TCT GAC AAG CTG GTG CGT CAC CAA CGT ACT CAC ACC
 CTT ACA CGG TTC AGA AAG TCG GCG AGA CTG TTC GAC CAC GCA GTG GTT GCA TGA GTG TGG
 E C G K S F S R S D K L V R H Q R T H T>

 550 560 570 580 590 600
 GGT AAA AAA ACT AGT GGC CAG GCC GGC CGC CGA AAT GAA ATG GGT GCT TCA GGA GAC ATG
 CCA TTT TTT TGA TCA CGG GTC CGG CGC GCG GCT TTA CTT TAC CCA CGA AGT CCT CTG TAC
 G K K T S G Q A G R R N E M G A S G D M>

 610 620 630 640 650 660
 AGG GCT GCC AAC CTT TGG CCA AGC CCT CTT GTG ATT AAG CAC ACT AAG AAG AAT ACC CCT
 TCC CGA CGG TTG GAA ACC GGT TCG GGA GAA CAC TAA TTC GTG TGA TTC TTA TCG GGA
 R A A N L W P S P L V I K H T K X N S P>

 670 680 690 700 710 720
 GCC TTG TCC TTG ACA GCT GAC CAG ATG GTC AGT GGC TTG GTG GAT GCT GAA CGG CCC ATG
 CGG AAC AGG AAC TGT CGA CTG GTC TAC CAG TCA CGG AAC AAC CTA CGA CTT GGC GGG TAC
 A L S L T A D Q M V S A L L D A E P P M>

 730 740 750 760 770 780
 ATC TAT TCT GAA TAT GAT CCT TCT AGA CCC TTC AGT GAA GCC TCA ATG ATG GGC TTA TTG

FIG. 9-1

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TAG ATA AGA CTT ATA CTA GGA AGA TCT GGG AAG TCA CTT CGG AGT TAC TAC CCG AAT AAC
 I Y S E Y D P S R F F S E A S M M G L L>
 790 800 810 820 830 840
 ACC AAC CTA GCA GAT AGG GAG CTG GTT CAT ATG ATC AAC TGG GCA AAG AGA GTG CCA GGC
 TGG TTG GAT CGT CTA TCC CTC GAC CAA GTA TAC TAG TTG ACC CGT TTC TCT CAC GGT CGG
 T N L A D R E L V H M I N W A K R V F G>
 850 860 870 880 890 900
 TTT GGG GAC TTG AAT CTC CAT GAT CAG GTC CAC CTT CTC GAG TGT GCC TGG CTG GAG ATT
 AAA CCC CTG AAC TTA GAG GTA CTA GTC CAG GTG GAA GAG CTC ACA CGG ACC GAC CTC TAA
 F G D L N L H D Q V H L L E C A W L E I>
 910 920 930 940 950 960
 CTG ATG ATT GGT CTC GTC TGG CGC TCC ATG GAA CAC CGG GGG AAG CTC CTG TTT GCT CCT
 GAC TAC TAA CCA GAG CAG ACC CGG AGG TAC CTT GTG GGC CCC TTC GAG GAC AAA CGA GGA
 L M I G L V W R S M E H P G K L L F A P>
 970 980 990 1000 1010 1020
 AAC TTG CTC CTG GAC AGG AAT CAA GGT AAA TGT GTG GAA GGC ATG GTG GAG ATC TTT GAC
 TTG AAC GAG GAC CTG TCC TTA GTT CCA TTT ACA CAC CTT CGG TAC CAC CTC TAG AAA CTG
 N L L D R N Q G K C V E G M V E I F D>
 1030 1040 1050 1060 1070 1080
 ATG TTG CTT GCT ACG TCA AGT CGG TTC CGC ATG ATG AAC CTG CAG GGT GAA GAG TTT GTG
 TAC AAC GAA CGA TGC AGT TCA GCC AAG GCG TAC TAC TTG GAC GTC CCA CTT CTC AAA CAC
 M L L A T S S R F R M M N L Q G E E F V>
 1090 1100 1110 1120 1130 1140
 TGC CTC AAA TCC ATC ATT TTG CTT AAT TCC GGA GTG TAC ACG TTT CTG TCC AGC ACC TTG
 AGC GAG TTT AGG TAG TAA AAC GAA TTA AGG CCT CAC ATG TGC AAA GAC AGG TCG TGG AAC
 C L K S I I L L N S G V Y T F L S S T L>
 1150 1160 1170 1180 1190 1200
 AAG TCT CTG GAA GAG AAG GAC CAC ATC CAC CGT GTC CTG GAC AAG ATC ACA GAC ACT TTG
 TTC AGA GAC CTT CTC TTC CTG GTG TAG GTG GCA CAG GAC CTG TTC TAG TGT CTG TGA AAC
 K S L E E K D H I R V L D K I T D T L>
 1210 1220 1230 1240 1250 1260
 ATC CAC CTG ATG GCC AAA GCT GGC CTG ACT CTG CAG CAG CAT CGC CGC CTC GCT CAG
 TAG GTG GAC TAC CGG TTT CGA CGG GAC TGA GAC GTC GTC GTC GCA GCG GCG GAT CGA GTC
 I H L M A K A G L T L Q Q Q H R R L A Q>
 1270 1280 1290 1300 1310 1320
 CTC CTT CTC ATT CTT TCC CAT ATC CGG CAC ATG AGT AAC AAA GGC ATG GAG CAT CTC TAC
 GAG GAA GAG TAA GAA AGG GTA TAG GCC GTG TAC TCA TTG TTT CCG TAC CTC GTA GAG ATG
 L L L I L S H I R H M S N K G M E H L Y>
 1330 1340 1350 1360 1370 1380
 AAC ATG AAA TGC AAG AAC GTT GTG CCC CTC TAT GAC CTG CTC CTG GAG ATG TTG GAT GCC
 TTG TAC TTT AGC TTC TTG CAA CAC CGG GAG ATA CTG GAC GAG GAC CTC TAC AAC CTC CGG
 N M K C K N V V P L Y D L L L E M L D A>
 1390 1400 1410 1420 1430 1440
 CAC CGC CTT CAT GCC CCA GCC AGT CGC ATG GGA GTG CCC CCA GAG GAG CCC AGC CAG ACC
 GTG GCG GAA GTA CGG GGT CGG TCA GCG TAC CCT CAC CGG GGT CTC CTC GGG TCG GTC TGG
 H R L H A P A S R M G V P P E E P S Q T>
 1450 1460 1470 1480 1490 1500
 CAG CTG GCC ACC ACC AGC TCC ACT TCA GCA CAT TCC TTA CAA ACC TAC TAC ATA CCC CGG
 GTC GAC CGG TGG TGG TCG AGG TGA AGT CGT GTA AGG AAT GTT TGG ATG ATG TAT GGG GGC
 Q L A T T S S T S A H S L Q T Y Y I P P>
 1510 1520 1530 1540 1550 1560

FIG. 9-2

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FIG. 9-3

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2290	2300	2310	2320	2330	2340
CTT TCC CAT ATC CGG CAC ATG AGT AAC AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC GAA ACG GTA TAG GCC GTG TAC TCA TTG TTT CCG TAC CTC GTA GAG ATG TTG TAC TTT ACG L S H I R H M S N K G M E H L Y N M K C>					
2350	2360	2370	2380	2390	2400
AAG AAC GTT GTG CCC CTC TAT GAC CTG CTC CTG GAG ATG TTG GAT GCC CAC CGC CTT CAT TTC TTG CAA CAC GGG GAG ATA CTG GAC GAG GAC CTC TAC AAC CTA CGG GTG GCG GAA GTA K N V V P L Y D L L E M L D A H R L H>					
2410	2420	2430	2440	2450	2460
GCC CCA GCC AGT CGC ATG GGA GTG CCC CCA GAG GAG CCC AGC CAG ACC CAG CTG GCC ACC CGG GGT CGG TCA CGG TAC CCT CAC GGG GGT CTC CTC GGG TCG GTC TGG GTC GAC CGG TGG A P A S R M G V P P E E P S Q T Q L A T>					
2470	2480	2490	2500	2510	2520
ACC AGC TCC ACT TCA GCA CAT TCC TTA CAA ACC TAC TAC ATA CCC CCG GAA GCA GAG GGC TGG TCG AGG TGA AGT CGT GTA AGG AAT GTT TGG ATG ATG TAT GGG GGC CTT CGT CTC CCG T S S T S A H S L Q T Y Y I P P E A E G>					
2530	2540	2550	2560	2570	2580
TTC CCC AAC ACG ATC GGG CGC GCC GAC GCG CTG GAC GAT TTC GAT CTC GAC ATG CTG GGT AAG GGG TTG TGC TAG CCC GCG CGG CTG CGC GAC CTG CTA AAG CTA GAG CTG TAC GAC CCA F P N T I G R A D A L D D F D L D M L G S D A L D D F>					
2590	2600	2610	2620	2630	2640
TCT GAT GCC CTC GAT GAC TTT GAC CTG GAT ATG TTG GGA AGC GAC GCA TTG GAT GAC TTT AGA CTA CGG GAG CTA CTG AAA CTG GAC CTA TAC AAC CCT TCG CTG CGT AAC CTA CTG AAA S D A L D D F D L D M L G S D A L D D F>					
2650	2660	2670	2680	2690	2700
GAT CTG GAC ATG CTC GGC TCC GAT GCT CTG GAC GAT TTC GAT CTC GAT ATG [TTA ATT AAC CTA GAC CTG TAC GAG CGG AGG CTA CGA GAC CTG CTA AAG CTA GAG CTA TAC AAT TAA TTG D L D M L G S D A L D D F D L D M L I N>					
2710	2720	2730			
TAC CCG TAC GAC GTT CCG GAC TAC GCT TCT TGA GAA TTC ATG GGC ATG CTG CAA GGC CTG ATG CGA AGA ACT CTT AAG Y P Y D V P D Y A S * E F>					

FIG. 9-4

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10 20 30 40 50 60
 GGA TCC GCC ACC ATG GCC CAG GCG GCC CTC GAG CCC GGG GAG AAG CCC TAT GCT TGT CGC
 CCT AGG CGG TGG TAC CGG GTC CGC CGG GAG CTC GGG CCC CTC TTC GGG ATA CGA ACA CGC
 G S A T M A Q A A L E P G E K P Y A C P>

 70 80 90 100 110 120
 GAA TGT GGT AAG TCC TTC AGT AGG AAG GAT TCG CTT GTG AGG CAC CAG CGT ACC CAC ACG
 CTT ACA CCA TTC AGG AAG TCA TCC CTA AGC GAA CAC TCC GTG GTC GCA TGG GTG TGC
 E C G K S F S R K D S L V R H Q R T H T>

 130 140 150 160 170 180
 GGT GAA AAA CCG TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGT CAG TCG GGG GAT CTT
 CCA CTT TTT GGC ATA TTT ACG GGT CTC ACY CGG TTT AGA AAA TCA GTC AGC CCC CTA GAA
 G E K P Y K C P E C G K S F S Q S G D L>

 190 200 210 220 230 240
 AGG CGT CAT CAA CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA GAA TGT CGC AAG
 TCC GCA GTA GTT GCG TGA GTA CGG CTC TTC GGT ATG TTT ACA GGT CTT ACA CGG TTC
 R R H Q R T H T G E K P Y K C P E C G K>

 250 260 270 280 290 300
 TCT TTC AGT GAT TGT CGT GAT CTT GCG AGG CAC CAA CGT ACT CAC ACC GGG GAG AAG CCC
 AGA AAG TCA CTA ACA GCA CTA GAA CGC TCC GTG GTT GCA TGA GTG TGG CCC CTC TTC GGG
 S F S D C R D L A R H Q R T H T G E K P>

 310 320 330 340 350 360
 TAT GCT TGT CCG GAA TGT GGT AAG TCC TTC TCT CAG ACC TCT CAC CTG GTG CGC CAC CAG
 ATA CGA ACA CGC CTT ACA CCA TTC AGG AAG AGA GTC TCG AGA GTG GAC CAC CGC GTG GTC
 Y A C P E C G K S F S Q S S H L V R H Q>

 370 380 390 400 410 420
 CGT ACC CAC ACG GGT GAA AAA CCG TAT AAA TGC CCA GAG TGC GGC AAA TCT TTT AGT GAC
 GCA TGG GTG TGC CCA CTT TTT GGC ATA TTT ACG GGT CTC ACY CGG TTT AGA AAA TCA CTG
 R T H T G E K P Y K C P E C G K S F S D>

 430 440 450 460 470 480
 TGC CGC GAC CTT GCT CGC CAT CAA CGC ACT CAT ACT GGC GAG AAG CCA TAC AAA TGT CCA
 ACY CGC CTG GAA CGA CGC GTA GTT GCG TGA GTA CGG CTC TTC GGT ATG TTT ACA GGT
 C R D L A R H Q R T H T G E K P Y K C P>

 490 500 510 520 530 540
 GAA TGT GGC AAG TCT TTC AGC CGC TCT GAC AAG CTG GTG CGT CAC CAA CGT ACT CAC ACC
 CTT ACA CGG TTC AGA AAG TCG CGC AGA CTG TTC GAC CAC GCA GTG GTT GCA TGA GTG TGG
 E C G K S F S R S D K L V R H Q R T H T>

 550 560 570 580 590 600
 GGT AAA AAA ACT AGT GGC CAG GCC GGC CGC CGA AAT GAA ATG GGT GCT TCA GGA GAC ATG
 CCA TTT TTT TGA TCA CGG GTC CGG CGG GCT TTA CTT TAC CCA CGA AGT CCT CTG TAC
 G K K T S G Q A G R R N E M G A S G D M>

 610 620 630 640 650 660
 AGG GCT GCC AAC CTT TGG CCA AGC CCT CTT GTG ATT AAG CAC ACT AAG AAG AAT AGC CCT
 TCC CGA CGG TTG GAA ACC GGT TCG GGA GAA CAC TAA TTC GTG TGA TTC TCA TCG GGA
 R A A N L W P S P L V I K H T K K N S F>

 670 680 690 700 710 720
 GCC TTG TCC TTG ACA GCT GAC CAG ATG GTC AGT GCC TTG GTG GAT GCT GAA CGG CCC ATG
 CGG AAC AGG AAC TGT CGA CTG GTC TAC CAG TCA CGG AAC AAC CTA CGA CTT GGC GGG TAC
 A L S L T A D Q M V S A L L D A E P P M>

 730 740 750 760 770 780
 ATC TAT TCT GAA TAT GAT CCT TCT AGA CCC TTC AGT GAA GCC TCA ATG ATG GGC TTA TTG

FIG. 10-1

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TAG ATA AGA CTT ATA CTA GGA AGA TCT GGG AAG TCA CTT CGG AGT TAC TAC CCG AAT AAC
 I Y S E Y D P S R P F S E A S M M G L >
 790 800 810 820 830 840
 ACC AAC CTA GCA GAT AGG GAG CTG GTT CAT ATG ATC AAC TGG GCA AAG AGA GTG CCA GGC
 TGG TTG GAT CGT CTA TCC CTC GAC CAA GTA TAC TAG TTG ACC CGT TTC TCT CAC GGT CGC
 T N L A D R E L V H M I N W A K R V P G >
 850 860 870 880 890 900
 TTT GGG GAC TTG AAT CTC CAT GAT CGT GTC CAC CTT CTC GAG TGT GCC TGG CTG GAG ATT
 AAA CCC CTG AAC TTA GAG GTA CTA GTC CAG GTG GAA GAG CTC ACA CGG ACC GAC CTC TAA
 F G D L N L H D Q V H L L E C A W L E I >
 910 920 930 940 950 960
 CTG ATG ATT GGT CTC GTC TGG CGC TCC ATG GAA CAC CCG GGG AAG CTC CTG TGT CCT CCT
 GAC TAC TAA CCA GAG CAG ACC GCG AGG TAC CTT GTG GGC CCC TTC GAG GAC AAA CGA GGA
 L M I G L V W R S M E H P G K L L F A P >
 970 980 990 1000 1010 1020
 AAC TTG CTC CTG GAC AGG AAT CAA GGT AAA TGT GTG GAA GGC ATG GTG GAG ATC TTT GAC
 TTG AAC GAG GAC CTG TCC TTA GTT CCA TTT ACA CAC CTT CCG TAC CAC CTC TAG AAA CTG
 N L L D R N Q G K C V E G M V E I F D >
 1030 1040 1050 1060 1070 1080
 ATG TTG CTT GCT ACG TCA AGT CGG TTC CGC ATG ATG AAC CTG CAG GGT GAA GAG TTT GTG
 TAC AAC GAA CGA TGC AGT TCA GCC AAG GCG TAC TAC TTG GAC GTC CCA CTT CTC AAA CAC
 M L L A T S S R F R M M N L Q G E E F V >
 1090 1100 1110 1120 1130 1140
 TGC CTC AAA TCC ATC ATT TTG CTT AAT TCC GGA GTG TAC ACG TTT CTG TCC AGC ACC TTG
 AC GAG TTT AGG TAG TAA AAC GAA TTA AGG CCT CAC ATG TGC AAA GAC AGG TCG TGG AAC
 C L K S I I L L N S G V Y T F L S S T L >
 1150 1160 1170 1180 1190 1200
 AAG TCT CTG GAA GAG AAG GAC CAC ATC CAC CGT GTC CTG GAC AAG ATC ACA GAC ACT TTG
 TTC AGA GAC CTT CTC TTC CTG GTG TAG GTG GCA CAG GAC CTG TTC TAG TGT CTG TGA AAC
 K S L E E K D H I H R V L D X I T D T L >
 1210 1220 1230 1240 1250 1260
 ATC CAC CTG ATG GCC AAA GCT GGC CTG ACT CTG CAG CAG CAT CGC CGC CTA GCT CAG
 TAG GTG GAC TAC CGG TTT CGA CGG GAC TGA GAC GTC GTC GTC GTC GAC GCG GCG GAT CGA GTC
 I H L M A K A G L T L Q Q Q H R R L A Q >
 1270 1280 1290 1300 1310 1320
 CTC CTT CTC ATT CTT TCC CAT ATC CGG CAC ATG AGT AAC AAA GGC ATG GAG CAT CTC TAC
 GAG GAA GAG TAA GAA AGG GTA TAG GCC GTG TAC TCA TTG TTT CCG TAC CTC GTC GAG ATG
 L L L I L S H I R H M S N K G M E H L Y >
 1330 1340 1350 1360 1370 1380
 AAC ATG AAA TGC AAG AAC GTT GTG CCC CTC TAT GAC CTG CTC CTG GAG ATG TTG GAT GCC
 TTG TAC TTT ACG TTC TTG CAA CAC GGG GAG ATA CTG GAC GAG GAC CTC TAC AAC CTA CGG
 N M K C K N V V P L Y D L L L E M L D A >
 1390 1400 1410 1420 1430 1440
 CAC CGC CTT CAT GCC CCA GCC AGT CGC ATG GGA GTG CCC CCA GAG GAG CCC AGC CAG ACC
 GTG GCG GAA GTA CGG GGT CGG TCA GCG TAC CCT CAC GGG GGT CTC CTC GGG TCG GTC TCG
 H R L H A P A S R M G V P P E E P S Q T >
 1450 1460 1470 1480 1490 1500
 CAG CTG GCC ACC ACC AGC TCC ACT TCA GCA CAT TCC TTA CAA ACC TAC TAC TAC TAC ATA CCC CGC
 GTC GAC CGG TGG TGG TCG AGG TGA AGT CGT GTA AGG AAT GTT TGG ATG ATG TAT GGG GGC
 Q L A T T S S T S A H S L Q T Y Y I P P >
 1510 1520 1530 1540 1550 1560

FIG. 10-2

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GAA GCA GAG GGC TTC CCC AAC ACG ATC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG
 CTT CGT CTC CCG AAG GGG TTG TGC TAG AGG AGA CCA CCG CCA CCG AGC CCG CCA CCA CCC
 E A E G F P N T I S S G G G G S G G G G G>

1570	1580	1590	1600	1610	1620
GGT GGT TCC ACT AGC GGC GGT GGC GGT GGC TCC TCT GGT GGC GGT GGC GGT TCT TCC AAT	CCA CCA AGG TGA TCG CCG CCA CCG CCA CCG AGG AGA CCA CCG CCA CCG CCA AGA AGG TTA	G G S T S G G G S S G G G G G S S N			

1630	1640	1650	1660	1670	1680
GAA ATG GGT GCT TCA GGA GAC ATG AGG GCT GCC AAC		CTT TGG CCA AGC CCT CTT GTG ATT			
CTT TAC CCA CGA AGT CCT CTG TAC TCC CGA CGG TTG		GAA ACC GGT TCG GGA GAA CAC TAA			
E M G A S G D M R A A N L W P S P L V I >					

1690	1700	1710	1720	1730	1740
AAG CAC ACT AAG RAG AAT AGC CCT GCC TTG TCC TTG		ACA GCT GAC CAG ATG GTC AGT GCC			
TTC GTG TGA TTC TTC TTA TCG GGA CGG AAC AGG AAC		TGT CGA CTG GTC TAC CAG TCA CGG			
K H T K N S P A L S L T A D Q M V S A >					

1750	1760	1770	1780	1790	1800
TTG TTG GAT GCT GAA CCG CCC ATG ATC TAT TCT GAA TAT GAT CCT TCT AGA CCC TTC AGT	AAC AAC CTA CGA CTT GGC GGG TAC TAG ATA AGA CTT ATA CTA GGA AGA TCT GGG AAG TCA	L L D A E P B M I V S E V D P S F P E			

1810	1820	1830	1840	1850	1860
GAA GCC TCA ATG ATG GGC TTA TTG ACC AAC CTA GCA GAT AGG GAG CTG GTT CAT ATG ATC	CTT CGG AGT TAC TAC CCG AAT AAC TGG TTG GAT CGT CTA TCC CTC GAC CAA GTA TAC TAG	F A S M M G L L T N L A D E F I V H M			

1870	1880	1890	1900	1910	1920
AAC TGG GCA AAG AGA GTG CCA GGC TTT GGG GAC TTG AAT CTC CAT GAT CAG GTC CAC CTT	TTG ACC CGT TTC TCT AAC CCG GGT CCC AAA CCC CTG AAC TTA GAG GTA CTA GTC CAG GTG GAA				
N H A L K P V D S E F R I M Y D C Q V H I D					

1930 1940 1950 1960 1970 1980
CTC GAG TGT GCC TGG CTG GAG ATT CTG ATG ATT GGT CTC GTC TGG CGC TCC ATG GAA CAC
GAG CTC ACA CGG ACC GAC CTC TAA GAC TAC TAA CCA GAG CAG ACC GCG AGG TAC CTT GTG

1990 2000 2010 2020 2030 2040
 CCG GGG AAG CTC CTG TTT GCT CCT AAC TTG CTC CTG GAC AGG AAT CAA GGT AAA TGT GTG
 GGC CCC TTC GAG GAC AAA CGA GGA TTG AAC GAG GAC CTG TCC TTA GTT CCA TTT ACA CAC

2050	2060	2070	2080	2090	2100
GAA GGC ATG GTG GAG ATC TTT GAC ATG TTG CTT GCT ACG TCA AGT CGG TTC CGC ATG ATG	CTT CCG TAC CAC CTC TAG AAA CTG TAC AAC GAA CGA TGC AGT TCA GCC AAG GCG TAC TAC				

2110	2120	2130	2140	2150	2160
AAC CTG CAG GGT GAA GAG TTT GTG TGC CTC AAA TCC ATC ATT TTG CTT AAT TCC GGA GTG					
TTG GAC GTC CCA CTT CTC AAA CAC ACG GAG TTT AGG TAG TAA AAC GAA TTA AGG CCT CAC					

2170	2180	2190	2200	2210	2220
TAC ACG TTT CTG TCC AGC ACC TTG AAG TCT CTG GAA GAG AAG GAC CAC ATC CAC CGT GTC					
ATG TGC AAA GAC AGG TCG TGG AAC TTC AGA GAC CTT CTC TTC CTG GTG TAG GTG GCA CAG					

2230	2240	2250	2260.	2270	2280
CTG GAC AAG ATC ACA GAC ACT TTG ATC CAC CTG ATG GCC AAA GCT GGC CTG ACT CTG CAG					
GAC CTG TTC TAG TGT CTG TGA AAC TAG GTG GAC TAC CGG TTT CGA CCG GAC TGA GAC GTC					

FIG. 10-3

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2290	2300	2310	2320	2330	2340
CAG CAG CAT CGC CGC CTA GCT CAG CTC CTT CTC ATT CTT TCC CAT ATC CGG CAC ATG AGT					
GTC GTC GTA GCG GCG GAT CGA GTC GAG GAA GAG TAA GAA AGG GTA TAG GCC GTG TAC TCA					
Q Q H R R L A Q L L L I L S H I R H M S>					
2350	2360	2370	2380	2390	2400
AAC AAA GGC ATG GAG CAT CTC TAC AAC ATG AAA TGC AAG AAC GTT GTG CCC CTC TAT GAC					
TTG TTT CCG TAC CTC GTA GAG ATG TTG TAC TTT ACG TTC TTG CAA CAC GGG GAG ATA CTG					
N K G M E H L Y N M K C K N V V P L Y D>					
2410	2420	2430	2440	2450	2460
CTG CTC CTG GAG ATG TTG GAT GCC CAC CGG CTT CAT GCC CCA GCC AGT CGC ATG GGA GTG					
GAC GAG GAC CTC TAC AAC CTA CGG GTG GCG GAA GTA CGG GGT CGG TCA GCG TAC CCT CAC					
L L L E M L D A H R L H A P A S R M G V>					
2470	2480	2490	2500	2510	2520
CCC CCA GAG GAG CCC AGC CAG ACC CAG CTG GCC ACC ACC AGC TCC ACT TCA GCA CAT TCC					
GGG GGT CTC CTC GGG TCG GTC TGG GTC GAC CGG TGG TGG TCG AGG TGA AGT CGT GTA AGG					
P P E E P S Q T Q L A T T S S T S A H S>					
2530	2540	2550	2560	2570	2580
TTA CAA ACC TAC TAC ATA CCC CCG GAA GCA GAG GGC TTC CCC AAC ACG ATC GGG CGC GCC					
AAT GTT TGG ATG ATG TAT GGG GGC CTT CGT CTC CCG AAG GGG TTG TGC TAG CCC CGG CGG					
L Q T Y Y I P P E A E G F P N T I G R A>					
2590	2600	2610	2620	2630	2640
GAC GCG CTG GAC GAT TTC GAT CTC GAC ATG CTG GGT TCT GAT GCC CTC GAT GAC TTT GAC					
CTG CGC GAC CTG CTA AAG CTA GAG CTG TAC GAC CCA AGA CTA CGG GAG CTA CTG AAA CTG					
D A L D D F D L D M L G S D A L D D F D L D M L G S D>					
2650	2660	2670	2680	2690	2700
CTG GAT ATG TTG GGA AGC GAC GCA TTG GAT GAC TTT GAT CTG GAC ATG CTC GGC TCC GAT					
GAC CTA TAC AAC CCT TCG CTG CGT AAC CTA CTG AAA CTA GAC CTG TAC GAG CGG AGG CTA					
L D M L G S D A L D D F D L D M L G S D>					
2710	2720	2730	2740	2750	2760
GCT CTG GAC GAT TTC GAT CTC GAT ATG TTA ATT AAC TAC CCG TAC GAC GTT CCG GAC TAC					
CGA GAC CTG CTA AAG CTA GAG CTA TAC AAT TAA TTG ATG GGC ATG CTG CAA GGC CTG ATG					
A L D D F D L D M L I N Y P Y D V P D Y>					
2770					
GCT TCT TGA GAA TTC					
CGA AGA ACT CTT AAG					
A S * E F>					

Pac1

FIG. 10-4

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(71) Applicant (*for all designated States except AT, US*): **NOVARTIS AG [CH/CH]; Lichtstrasse 35, CH-4056 Basel (CH)**.

(71) Applicant (*for AT only*): **NOVARTIS-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B. H. [AT/AT]; Brunner Strasse 59, A-1230 Vienna (AT)**.

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(72) Inventors; and

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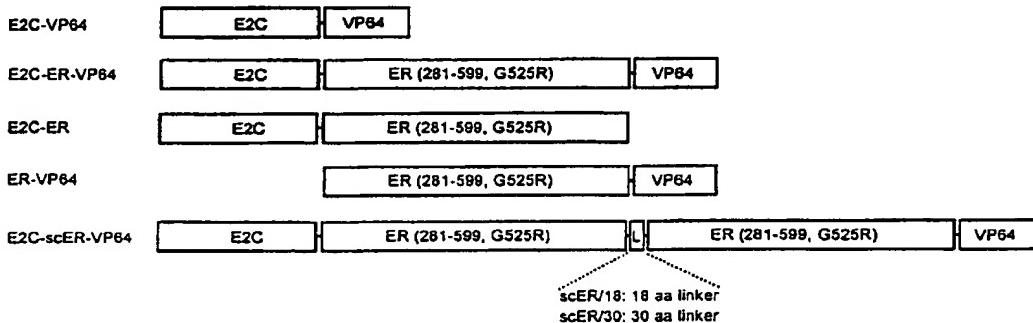
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- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **REGULATION OF GENE EXPRESSION USING SINGLE-CHAIN, MONOMERIC, LIGAND DEPENDENT POLYPEPTIDE SWITCHES**



3
A3
WO 02/006463

(57) Abstract: Single chain, monomeric polypeptide gene switches are provided. The gene switches include ligand binding domains and at least one functional domain. Preferred functional domains are DNA binding domains and transcriptional regulating domains. Methods of regulating gene function using the switches are also provided.

INTERNATIONAL SEARCH REPORT

Inte

nal Application No

PCT/EP 01/08190

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/62 C12N15/63 C07K14/47 C07K14/72

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, SEQUENCE SEARCH, BIOSIS, EPO-Internal

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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